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The Oxidation of β-(3-Indolyl)propionic Acid and γ-(3-Indolyl)-*n*-butyric Acid by Peroxidase and Mn^{2+}

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Wagenknecht & Burris (1950) have shown that both β-(3-indolyl)propionic acid (IPA) and γ-(3-indolyl)-*n*-butyric acid (IBA) are oxidized by extracts of waxpod bean root (*Phaseolus vulgaris* L.) and of pea epicotyls (*Pisum sativum* L.), though the rate of oxidation of these compounds was less than that of 3-indolylacetic acid (IAA). With waxpod bean-root extracts the presence of small amounts of Mn^{2+} stimulated the oxidation of IPA and IBA, but with pea epicotyl extracts this effect of Mn^{2+} was observed only after the extract had been dialysed. Kenten (1955) reported that the direct oxidase action of peroxidase preparations on IAA was increased in the presence of certain monophenols, maleic hydrazide or Mn^{2+}. During this work it was found that horseradish peroxidase preparations also catalyse the oxidation of IPA and IBA by oxygen, but only in the presence of Mn^{2+}. These observations are recorded here, partly on account of the interest in systems which may be responsible for the metabolism of substances with growth hormone properties, and partly because they suggest that the oxidation of IPA and IBA by the plant extracts used by Wagenknecht & Burris (1950) may have been due to the presence of peroxidase and Mn^{2+} in the extracts.

MATERIALS AND METHODS

Glucose oxidase. A commercial preparation obtained from the Sigma Chemical Co., St Louis, U.S.A. 'Glucose oxidase, crude on inert base', was suspended in 0·2 M phosphate pH 6 (20 mg./ml.) and centrifuged. The clear yellow supernatant was used without further purification.

Peroxidase and catalase preparations. These were as described by Kenten (1955). In the text the peroxidase activity of some preparations is given as enzyme units (E.U.) (Keilin & Hartree, 1951). With a dry preparation the number of E.U. = P.N. × wt. of the preparation in g., where P.N. = purpurogallin number. For each experiment the catalase preparation ($k_1' = 2{\cdot}2 \times 10^6 M^{-1} sec.^{-1}$) was suspended (10 mg./ml.) in 0·02 M phosphate pH 6 and the insoluble material removed by centrifuging.

IPA and IBA solutions. Unless otherwise stated, IPA or IBA was brought into solution by warming and adding 5% (w/v) NH_3. After cooling, the solutions were adjusted to pH 6–7 with NH_3 and stored in the dark at 0°. Fresh solutions were prepared weekly.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 and KOH; pyrophosphate buffers from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$; citrate buffers from solutions of citric acid and sodium citrate.

Manometric experiments. These were carried out with Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre well unless otherwise stated. The formation of CO_2 was measured by Warburg's direct method (Dixon, 1943).

RESULTS

Manometric studies of the oxidation of IPA by horseradish-peroxidase preparations and manganous ions

In phosphate at pH 6 horseradish-peroxidase preparations catalyse the oxidation of IPA by oxygen provided that Mn^{2+} is present. Before the oxidation begins there is often a lag period. The lag period is variable and is at least partially dependent on the age of the IPA solution and the light intensity prevailing at the time the experiment is carried out.

The lag period. For most of the present work warm water and NH_3 were used to neutralize and assist solution of the IPA. A lag period was usually found when such solutions were used immediately after preparation. With one single exception among a number of experiments a lag period was obtained when neutral solutions of IPA were prepared by using NH_3 and keeping the temperature below 5°. A typical experiment with an IPA solution prepared in this way is shown in Fig. 1. Reaction mixtures consisted of: in the main vessel $3{\cdot}3 \times 10^{-3}$ M IPA and $3{\cdot}3 \times 10^{-5}$ M-$MnSO_4$ in $6{\cdot}7 \times 10^{-2}$ M phosphate at pH 6, and where present $1{\cdot}3 \times 10^{-5}$ M-H_2O_2. After equilibration 100 μg. horseradish peroxidase P.N. 940 was added from the side arm. One experiment was carried out immediately the IPA solution was prepared and a second after storing the solution at 0° in darkness for 24 hr. In the absence of Mn^{2+} or peroxidase the oxygen uptake was negligible, but where both these factors were present a rapid uptake commenced after a lag period. The lag period was reduced by keeping the IPA solution at 0° for 24 hr. It was abolished when hydrogen peroxide was added to the reaction

mixtures, suggesting that the reaction depends on the formation of peroxide. In the absence of added H_2O_2, once the oxygen uptake began it proceeded at the same rate as that found in the presence of added H_2O_2. Working under conditions similar to those of Fig. 1, lag periods of up to 2·5 hr. have been observed, but 2–3 days storage of the IPA solution at 0° often considerably shortened the lag period. It was also found that exposure of the IPA solution to bright light reduced the lag period. In one experiment the Warburg vessels containing the reaction mixtures were exposed to sunlight for 5 min. before equilibration. No lag was observed with these reaction mixtures, whereas a lag period of 2 hr. was found with control mixtures protected from sunlight. Further control experiments showed that a 5 min. exposure to sunlight of the IPA solution in phosphate at pH 6 was sufficient to abolish or bring about a large reduction in the lag period.

Unless otherwise stated, most of the present work was carried out under conditions where only brief lag periods were obtained. This was achieved either by using IPA solutions which had been aged at 0° in darkness for 3–4 days, or by adding small amounts of hydrogen peroxide to the reaction mixtures. In no case was any significant difference in the rate of oxygen uptake observed whether added hydrogen peroxide, light or ageing of the IPA solution was used to reduce the lag period.

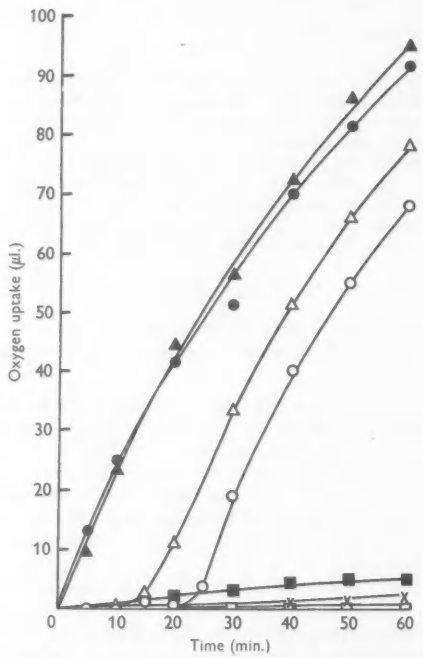

Fig. 1. The oxidation of IPA by a horseradish-peroxidase preparation P.N. 940 plus Mn^{2+}. Peroxidase and Mn^{2+} with: ○, freshly prepared IPA; △, IPA kept at 0° for 24 hr.; ●, freshly prepared IPA + H_2O_2; ▲, IPA kept at 0° for 24 hr. + H_2O_2. Control experiments: from the complete system of IPA, Mn^{2+}, peroxidase and H_2O_2 the following were omitted: ×, Mn^{2+}; □, peroxidase + H_2O_2, or Mn^{2+} + H_2O_2; ■, peroxidase.

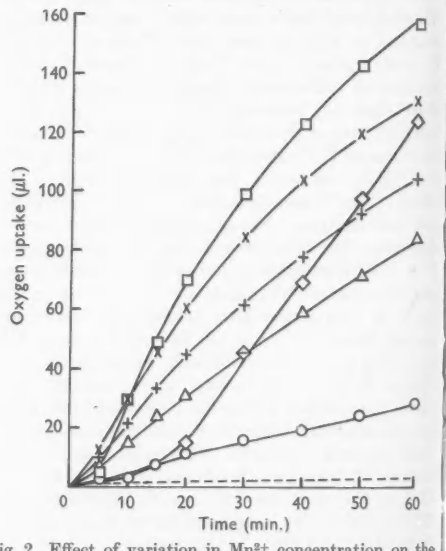

Fig. 2. Effect of variation in Mn^{2+} concentration on the rate of oxidation of IPA by horseradish peroxidase P.N. 940. Experiments in $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6, $6{\cdot}7 \times 10^{-3}$M IPA, 30 μg. horseradish peroxidase P.N. 940 and varying amounts of $MnSO_4$, ○, 5×10^{-6}M; △, 10^{-5}M; +, 3×10^{-5}M; ×, 10^{-4}M; □, 3×10^{-4}M; ◇, 10^{-3}M. Control experiments omitting peroxidase, Mn^{2+} or IPA gave O_2 uptakes less than that shown by the dotted line.

Effect of varying the concentration of Mn^{2+}. The effect of varying the concentration of Mn^{2+} on the rate of oxidation of IPA solutions, previously aged at 0°, by horseradish peroxidase is shown in Fig. 2. With increasing amounts of Mn^{2+} up to about 10^{-4}M the initial rate of oxygen uptake increases, but with further increases in Mn^{2+} there is a lag period before the oxidation begins. The length of the lag period varied in different experiments; there was a tendency for the lag period to increase with increase in Mn^{2+} from 3×10^{-4} to 10^{-3}M, but

such a regular effect was not always found. At the end of the experiment the complete reaction mixtures were coloured brownish yellow, the most intense coloration being found in the mixtures with the largest oxygen uptake. The results show that measurable rates of oxidation of IPA by peroxidase are obtained in the presence of amounts of Mn^{2+} as low as 5×10^{-6}M (i.e. 0·25 μg./ml.). In order to avoid a lag period induced by the presence of too high a concentration of Mn^{2+}, most of the present work has been carried out with Mn^{2+} concentrations of 10^{-4}M.

Effect of various metals. The cations Co^{2+}, Ni^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} were tested at concentrations of 10^{-3} and 10^{-4}M in reaction mixtures consisting of $6{\cdot}7 \times 10^{-3}$M IPA, 30 μg. horseradish peroxidase P.N. 940 and $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6. No significant oxygen uptake was obtained in these experiments in a 1 hr. period. Control experiments made simultaneously with mixtures containing 10^{-4}M-$MnSO_4$ gave uptakes of 134–142 μl. oxygen in 1 hr.

Effect of pH. This was tested in reaction mixtures consisting of: in the main vessel $6{\cdot}7 \times 10^{-3}$M IPA, 10^{-4}M-$MnSO_4$ and 10^{-4}M-H_2O_2 in buffer $3{\cdot}3 \times 10^{-2}$M with respect to acetate and phosphate; after equilibration 30 μg. horseradish peroxidase P.N. 940 were added from the side arm. The maximum rate of oxygen uptake was obtained at pH 6, the rates at pH 5 and 7 being respectively 80 and 65 % of that at pH 6, while at pH 4 no oxygen uptake was observed in 1 hr. The oxygen uptakes obtained with reaction mixtures, in the range pH 4–7, from which either Mn^{2+} or peroxidase was omitted were negligible. When freshly prepared IPA was used and hydrogen peroxide omitted from the reaction mixtures it was found that the lag period decreased with increase in pH value. In one typical experiment the length of the lag period was 30 min. at pH 7 and 100 min. at pH 6, but at pH 5 the reaction had not started after 180 min.

Effect of catalase. When horseradish peroxidase (30 μg. P.N. 940) was added to reaction mixtures containing $6{\cdot}7 \times 10^{-3}$M IPA, 10^{-4}M-$MnSO_4$ and $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6, the presence of 0·1–0·5 mg. catalase preparation caused an increase in the lag period, after which the oxygen uptake commenced, though with the larger amounts of catalase at a reduced rate. By the use of flasks with double side arms it was shown that the addition of larger amounts of catalase (2 mg.), after the reaction had started, brought about a total inhibition (Fig. 3).

Effect of varying the concentration of peroxidase. The effect of varying the amount of a horseradish-peroxidase preparation of P.N. 500 on the rate of oxidation of IPA in the presence of Mn^{2+} is shown in Table 1. The results show that the rate of uptake increases with increase in amount of peroxidase until a maximum rate is reached at about 20 μg. peroxidase preparation/ml.

Experiments with peroxidase preparations of different purity. A number of peroxidase preparations of varying degrees of purity were tested for their capacity to catalyse the oxidation of IPA by

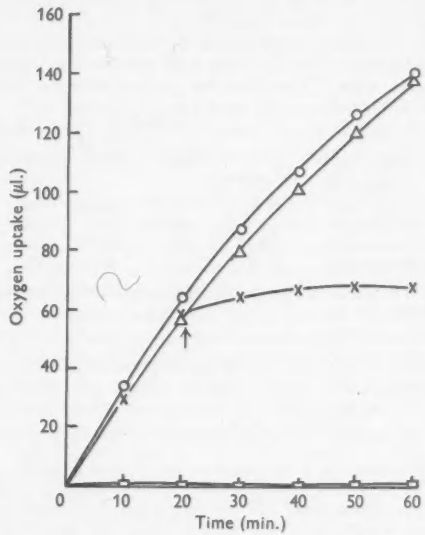

Fig. 3. Effect of catalase on the oxidation of IPA by horseradish peroxidase P.N. 940 plus Mn^{2+}. Reaction mixtures consisted of; in the main vessel $6{\cdot}7 \times 10^{-3}$M IPA, 10^{-4}M-$MnSO_4$ in $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6 and where present 2 mg. catalase preparation. After equilibration 30 μg. horseradish peroxidase was added from the side arm and 21 min. later 2 mg. catalase, or 2 mg. catalase previously heated 10 min. at 100°, was added from the second side arm. ○, Catalase omitted; □, catalase present initially; ×, catalase added at ↑; △, heated catalase added at ↑.

Table 1. *Effect of varying the concentration of peroxidase*

Reaction mixtures consisted of: in the main vessel $6{\cdot}7 \times 10^{-3}$M IPA and 10^{-4}M-$MnSO_4$ in $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6; after equilibration various amounts of horseradish peroxidase P.N. 500 were added from the side arm.

Peroxidase added (μg.)	Uptake of O_2 (μl. in 1 hr.)
2	27
4	50·5
8	87·5
16	106·5
32	122
64	145
128	143·5

oxygen in the presence of Mn^{++}. The results (Table 2) show that the various preparations do not differ appreciably in activity, suggesting that the observed oxidation of IPA is due to peroxidase.

Table 2. *Oxidation of IPA by different peroxidase preparations in the presence of* Mn^{2+}

Reaction mixtures consisted of: peroxidase preparation equivalent to 0·0025, 0·005 or 0·05 E.U., $6{\cdot}7 \times 10^{-3}$M IPA and 10^{-4}M-$MnSO_4$ in phosphate at pH 6; after equilibration the peroxidase was added from the side arm. Blank uptakes when IPA or Mn^{2+} was omitted were less than 4 μl. in 1 hr.

		Uptake of O_2 (μl. in 1 hr.)		
E.U. present		0·0025	0·005	0·05
Preparation	P.N.			
Turnip root	70	51·5	96	155
Horseradish	500	52	95	138
Horseradish	940	52	84	144
Horseradish	1040*	42	80	134

* The purity of this preparation is about 90% (Kenten & Mann, 1953).

Table 3. *Effect of heating the horseradish-peroxidase preparation of* P.N. 500 *on (i) the oxidation of IPA by oxygen in the presence of* Mn^{2+}, *and (ii) the oxidation of a pyrogallol by hydrogen peroxide*

Peroxidase (P.N. 500) solutions were heated in a boiling-water bath for 0, 5, 10, and 15 min. The activity of these solutions was tested with pyrogallol and H_2O_2 under the fixed conditions of the purpurogallin test and manometrically with IPA in reaction mixtures consisting of $6{\cdot}7 \times 10^{-3}$M IPA, 10^{-4}M-$MnSO_4$ and 10^{-5}M-H_2O_2 in $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6 with 10 μg. of the heated peroxidase.

Time of heating (min.)	Loss in activity (%)	
	IPA	Pyrogallol
5	44	50
10	68	75
15	81·5	89

Effect of heating the peroxidase preparation of P.N. 500. The results of Table 3 show that rather prolonged heating in the neighbourhood of 100° is necessary to inactivate the peroxidase. The fall in activity towards both IPA and pyrogallol brought about by heating the preparation is of the same order and supports the previous suggestion that the oxidation of IPA is due to peroxidase.

Effect of phenols and maleic hydrazide. Certain monophenols, resorcinol and maleic hydrazide stimulate, but polyphenols such as pyrogallol and catechol inhibit, the peroxidase-catalysed oxidation of IAA by oxygen (Kenten, 1955). Accordingly the effect of these substances was tested in reaction mixtures consisting of the phenol or maleic hydrazide, $6{\cdot}7 \times 10^{-3}$M IPA, 30 μg. horseradish peroxidase P.N. 940 and $6{\cdot}7 \times 10^{-2}$M phosphate at pH 6. The peroxidase was added from the side arm after equilibration. No significant oxygen uptake was obtained in 1 hr. with the phenols at 10^{-3}, 10^{-4} or 10^{-5}M or with maleic hydrazide at 5×10^{-3}M. When the reaction mixtures also contained 10^{-4}M-$MnSO_4$ no oxygen uptake was obtained in the presence of 10^{-4}M pyrogallol or catechol. However, in the presence of Mn^{2+}, although 10^{-2} and 10^{-3}M phenol inhibited, a slight stimulation of the oxygen uptake was observed with 10^{-4}M phenol (Table 4). By the use of reaction mixtures and conditions similar to those of Fig. 3, it was shown that the addition of catechol or pyrogallol (10^{-4}M final concentration) from the second side arm, 20 min. after the reaction between IPA, Mn^{2+} and peroxidase had started, brought about complete inhibition (Table 4).

Table 4. *Effect of phenols on the oxidation of IPA by peroxidase plus* Mn^{2+}

Reaction mixtures were similar to those of Fig. 3 except that catalase was replaced by catechol, or pyrogallol, or phenol.

Components present with IPA and peroxidase	Uptake of O_2 (μl.)	
	20 min.	60 min.
Mn^{2+}	58·5	133
Mn^{2+} + phenol 10^{-2}M	—	21
Mn^{2+} + phenol 10^{-3}M	—	92·5
Mn^{2+} + phenol 10^{-4}M	—	163
Mn^{2+} + phenol 10^{-5}M	—	137
Mn^{2+}, catechol 10^{-4}M added at 20 min.	58·5	61·5
Mn^{2+}, pyrogallol 10^{-4}M added at 20 min.	54	59

Effect of pyrophosphate and citrate. It has been shown previously (Kenten, 1955) that citrate and pyrophosphate strongly inhibit the activating effect of Mn^{2+} on the peroxidase catalysed oxidation of IAA by oxygen. It has been found that citrate and pyrophosphate at pH 6 also inhibit the reaction between IPA, Mn^{2+} and peroxidase. By the use of reaction mixtures and conditions similar to those of Fig. 3, except that the initial phosphate concentration was $3{\cdot}3 \times 10^{-2}$M, it was shown that an addition of pyrophosphate or citrate at pH 6 ($3{\cdot}3 \times 10^{-2}$M final concentration) from the second side arm reduced the subsequent rate of oxygen uptake by 95 and 75% respectively. Control experiments showed that an addition of phosphate was without effect. The effect of pyrophosphate and citrate is presumably due to their capacity of forming complex ions with Mn^{2+}.

Course of the oxidation. When reaction mixtures containing 200 μg. horseradish peroxidase P.N. 500, 10^{-4}M-$MnSO_4$, $3{\cdot}3 \times 10^{-5}$M-H_2O_2 (0·1 μmole) and varying amounts of IPA in $6{\cdot}7 \times 10^{-2}$M

phosphate at pH 6 were used, it was found that the total oxygen uptake varied with the initial IPA concentration. Thus in one experiment with 2·5, 5 and 10 μmoles IPA, the consumption of oxygen ceased after 4 hr. when the total uptake had reached values of 0·70, 0·67 and 0·55 μmole oxygen/mole IPA. With 5 μmoles IPA, increasing the peroxidase concentration sixfold increased the total oxygen uptake by only 8%. In these experiments, using Warburg's direct method, no carbon dioxide formation was detected.

Oxidation of IPA by horseradish-peroxidase preparations plus hydrogen peroxide. Colorimetric tests suggested that the oxidation of IPA by hydrogen peroxide was catalysed by horseradish peroxidase, with the formation of a yellow oxidation product. This was proved manometrically by using the glucose-oxidase system as a source of hydrogen peroxide:

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} C_6H_{12}O_7 + H_2O_2.$$

In the presence of catalase the hydrogen peroxide formed is decomposed and an apparent oxygen uptake of 0·5 mole oxygen/mole glucose is observed. If, however, a peroxidase system is present which uses the hydrogen peroxide for a secondary or coupled oxidation then an increase in the oxygen uptake is observed which may reach 1 mole oxygen/mole glucose if all the hydrogen peroxide produced is used in the coupled oxidation.

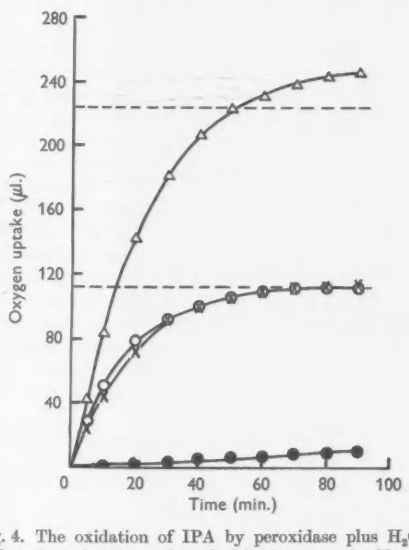

Fig. 4. The oxidation of IPA by peroxidase plus H_2O_2. Reaction mixtures as described in the text. △, Glucose + glucose oxidase + IPA + peroxidase + catalase; ×, peroxidase omitted; ○, IPA, or IPA + peroxidase, omitted; ●, glucose omitted.

Basal reaction mixtures consisted of: in the main vessel, 0·3 ml. glucose oxidase, 30 μg. catalase and 0·2 ml. 0·05 M glucose in 0·067 M phosphate at pH 6. The complete reaction mixtures contained, in addition, 30 μg. horseradish peroxidase P.N. 940 and 0·75 ml. 0·02 M IPA. The glucose was added from the side arm after equilibration. Control reaction mixtures were similar to the complete mixtures except that glucose, IPA or peroxidase was omitted.

The results (Fig. 4) show that in the absence of peroxidase or IPA the total oxygen uptake corresponded to 0·5 mole oxygen/mole glucose. In the presence of both peroxidase and IPA the total oxygen uptake was slightly greater than double this value, being about 1·1–1·2 mole oxygen/mole glucose. The 0·1–0·2 mole oxygen in excess of that theoretically required could be accounted for if peroxidase has a slight direct oxidase action under the conditions used. At the end of the experiment the complete reaction mixture was brownish yellow, indicating that oxidation of the IPA had taken place. The results suggest that the hydrogen peroxide produced by the glucose-oxidase system is used by the peroxidase to oxidize IPA.

Manometric studies of the oxidation of IBA by horseradish-peroxidase preparations and manganous ions. The few experiments which have been carried out with IBA suggest that, as with IPA, it is directly oxidized by the horseradish-peroxidase preparations only when Mn^{2+} is also present, and that monophenols cannot replace Mn^{2+} in this reaction. With reaction mixtures consisting of $6{\cdot}7 \times 10^{-3}$ M IBA, 10^{-4} M-$MnSO_4$ and 30 μg. horseradish peroxidase P.N. 940 in $6{\cdot}7 \times 10^{-2}$ M phosphate at pH 6, oxygen uptakes of 80–88 μl. in 1 hr. were obtained. The rate of oxidation of IBA is therefore less than that of IPA, since with this latter substance under similar conditions oxygen uptakes of about 140 μl. in 1 hr. were obtained. When either peroxidase or Mn^{2+} was omitted the oxygen uptake was negligible. In contrast to IPA, the oxidation of freshly prepared IBA solutions was found to proceed after only a brief lag period.

DISCUSSION

The results of the present work show that highly purified horseradish-peroxidase preparations catalyse the oxidation of IPA by oxygen in the presence of Mn^{2+}. With freshly prepared neutral IPA solutions the reaction is usually preceded by a lag period whose length may vary from a few minutes to 2·5 hr. The lag period can be reduced by ageing the IPA solution at 0°, exposing it to bright

light, or by adding very small amounts of hydrogen peroxide. Thus the variability of the length of the lag period could be partly due to differences in the light intensity in the laboratory when the different experiments were carried out. Since small amounts of added hydrogen peroxide abolish the lag period it is suggested that peroxides arise in IPA solutions when they are stored or exposed to light by autoxidation or photo-oxidation. Since IPA solutions absorb ultraviolet but not visible light such photo-oxidation is presumably brought about by ultraviolet light. The addition of catalase stops the reaction, suggesting that the reaction is accompanied by and depends on the formation of hydrogen peroxide. Although, under the conditions used, the direct oxidation of IPA by peroxidase depends on the presence of Mn^{2+}, it has also been shown that peroxidase readily catalyses the oxidation of IPA by hydrogen peroxide in the absence of Mn^{2+}. It would appear, therefore, that in the direct oxidase reaction Mn^{2+} may play a part in the production of hydrogen peroxide. Since at pH 6 IPA solutions consumed negligible amounts of oxygen whether Mn^{2+} was present or not it is clear that Mn^{2+} does not act by catalysing the autoxidation of IPA with the production of hydrogen peroxide.

Similar direct oxidase reactions have been described in which the oxidations of dihydroxymaleic acid (Swedin & Theorell, 1940), certain other dicarboxylic acids (Kenten & Mann, 1953), phenylacetaldehyde (Kenten, 1953) and IAA (Kenten, 1955) by oxygen are catalysed by peroxidase. The mechanism of this type of reaction is still largely a matter of discussion. Lemberg & Legge (1949) suggested a tentative mechanism for the dihydroxymaleic acid reaction, but it has been criticized by Theorell (1951) and Chance (1951) on the grounds that it does not account for the fact that 1 mole oxygen is consumed per mole of dihydroxymaleic acid. Chance (1952) has suggested that a Mn^{2+}-activated peroxidase–peroxide complex is the catalyst for the oxidase reaction with dihydroxymaleic acid, peroxidase and Mn^{2+}, while Kenten & Mann (1953) obtained evidence that the oxidation of certain dicarboxylic acids by peroxidase and Mn^{2+} was dependent on manganese oxidation.

In the present work it was found that neither hydrated MnO_2 nor manganipyrophosphate oxidized IPA rapidly at pH 6, and, if a manganese oxidation–reduction cycle plays a part in the oxidation of IPA, the oxidized manganese must react with an intermediate product.

In contrast to the results obtained by Kenten (1955), who found that the peroxidase-catalysed oxidation of IAA by oxygen was increased in the presence of certain monophenols, maleic hydrazide or Mn^{2+}, in the present work peroxidase did not catalyse the oxidation of IPA unless Mn^{2+} was also present, and monophenols, or maleic hydrazide, were not capable of replacing Mn^{2+} in this reaction. A stimulation of the oxidation of IPA by peroxidase and Mn^{2+} was observed when 10^{-4}M phenol was also present but the effect was small, and lower (10^{-5}M) or higher (10^{-3}–10^{-2}M) concentrations of phenol were without significant effect or were inhibitory. It has been demonstrated that polyphenols (Kenten, 1955), when present in low concentration, strongly inhibit the peroxidase-catalysed oxidation of IAA by oxygen. In the present work it has been shown that pyrogallol and catechol have a similar effect on the oxidation of IPA by peroxidase and Mn^{2+}. The inhibiting effect of the polyphenols appears to be most easily explained by assuming that they are more readily oxidized by peroxidase than is IPA and that they therefore compete successfully for the hydrogen peroxide formed.

Although comparatively few experiments were carried out with IBA the results obtained were essentially similar to those found with IPA, except that the oxidation proceeded with only a brief lag period.

Since the oxidation of IPA and IBA proceed at concentrations of peroxidase and Mn^{2+} commonly encountered in plant extracts, it is probable that the oxidation of these compounds observed by Wagenknecht & Burris (1950) was due to the presence of peroxidase and Mn^{2+} in the plant extracts. In support of this suggestion it has been found that the oxidation of IBA by peroxidase and Mn^{2+} proceeds at a slower rate than that of IPA, and the oxidation of this latter compound takes place without the formation of significant amounts of carbon dioxide. These properties agree with those reported for bean root and pea epicotyl preparations by Wagenknecht & Burris (1950). In this connexion it may be noted that citrate inhibits the peroxidase-catalysed oxidation of IPA by oxygen in the presence of Mn^{2+}, and although Tang & Bonner (1947), working in citrate–phosphate, failed to demonstrate the oxidation of IPA by pea-epicotyl preparations, Wagenknecht & Burris (1950), using phosphate buffers and similar preparations, found that IPA was readily oxidized.

Since neither IPA nor IBA is a known plant metabolite the present reaction is probably without physiological significance. It is of interest, however, that peroxidase systems are connected with the metabolism of other indole compounds, namely tryptophan (Wiltshire, 1953) and IAA (e.g. Galston, Bonner & Baker, 1953; Kenten, 1955), which are known to be plant metabolites. Since Mann & Smithies (1955) have shown that peroxidase catalyses the oxidation of indole by hydrogen peroxide, it is likely that with the other compounds the indole nucleus is the site of peroxidase attack.

SUMMARY

1. The oxidation of IPA and IBA by oxygen is catalysed by highly purified horseradish peroxidase preparations in the presence of Mn^{2+}.

2. The mechanism of the reaction has not been elucidated but the formation of hydrogen peroxide as an obligatory intermediate is suggested by the inhibiting effect of catalase.

3. It is suggested that the previously reported oxidation of IPA and IBA by plant extracts may have been due to the presence of peroxidase and Mn^{2+} in the extracts.

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Absorption and Metabolism of [^{14}C]Pyrethroids by the Adult Housefly, *Musca domestica* L., *in vivo*

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The pyrethrins represent the most important group of pyrethroid insecticides of natural origin and are widely used for the control of various insect pests, particularly houseflies. The flowers of the plant *Chrysanthemum cinerariaefolium* provide a rich source of the natural insecticide, e.g. by extraction with light petroleum (LaForge & Haller, 1935). The natural pyrethrins are a mixture of the four principal esters (indicated in Fig. 1) and their stereoisomers (Harper, 1949).

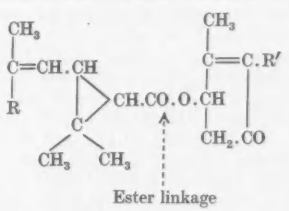

Fig. 1. Pyrethroids of natural origin.

Ester	Structure
Pyrethrin I	$R=CH_3$; $R'=-CH_2.CH{:}CH.CH{:}CH_2$
Cinerin I	$R=CH_3$; $R'=-CH_2.CH{:}CH{:}CH_3$
Pyrethrin II	$R=CO.O.CH_3$; $R'=-CH_2.CH{:}CH.CH{:}CH_2$
Cinerin II	$R=CO.O.CH_3$; $R'=-CH_2.CH{:}CH.CH_3$

In this paper the esters derived from the *cyclo*propanemonocarboxylic acid (chrysanthemic acid, $R=CH_3$) and the *cyclo*pentenone alcohol are called chrysanthemic esters, whereas those derived from the *cyclo*propanedicarboxylic acid monomethyl ester (pyrethric acid, $R=CO_2Me$) are called the pyrethric esters. Several insecticidally active analogues and homologues of the chrysanthemic esters have been synthesized in recent years (Schechter, Green & LaForge, 1949; Crombie, Edgar, Harper, Lowe & Thompson, 1950). Allethrin is the name approved by the U.S. Interdepartmental Committee on Pest Control for the synthetic allyl homologue ($R=CH_3$; $R'=CH_2.CH{:}CH_2$) of cinerin I and is an economically important ester. It is more or less toxic than the natural esters to various insect species (Gersdorff, 1949; Bottger, 1951) and appears to have greater stability (Blackith, 1952). The toxicity of the natural pyrethroids to insects is enhanced by the prior or simultaneous application of suitable synergists, particularly certain compounds containing the methylenedioxyphenyl group (Schroeder, Jones & Lindquist, 1948; Chamberlain, 1950; Kerr, 1951). The synergists are themselves relatively non-insecticidal. The toxicity of allethrin

is similarly but less effectively enhanced by these compounds (Gersdorff, Nelson & Mitlin, 1951, 1953). Chamberlain (1950) studied the synergism between the natural pyrethroids and the synergist piperonyl butoxide, which contains principally α-[2-(2-butoxyethoxy)ethoxy]-4:5-methylenedioxy-2-propyltoluene. He concluded that the synergism did not involve physical factors such as an increased permeability to the insecticide of the insect cuticle, and suggested that the synergist might inhibit a pyrethroid detoxication mechanism of the insect. He considered that lipase action was a likely detoxication mechanism because of its wide distribution in insect tissue and the ester linkage (Fig. 1) of the pyrethroids. His experiments to demonstrate this were inconclusive. The experiments described in this paper were designed to determine the nature and extent of pyrethroid metabolism by the adult housefly, *Musca domestica* L. *in vivo* and to determine whether or not this metabolism was inhibited in the presence of the synergist piperonyl cyclonene [a mixture of 3-*iso*pentyl-6-ethoxycarbonyl-5-(3:4-methylenedioxyphenyl)-2-*cyclo*hexen-1-one and 3-*iso*pentyl-5-(3:4-methylenedioxyphenyl)-2-*cyclo*hexen-1-one]. Preliminary studies were made by one of us (F. P. W. W.) in the Insect Toxicology Laboratory of the University of California, Berkeley, in the spring of 1952, using [^{14}C]pyrethroids prepared biosynthetically by Pellegrini, Miller & Sharpless (1952), and they have been briefly reported (Wineringham, 1952*a*). Later studies were made with fully synthetic [^{14}C]allethrin (see below). In all cases the labelled insecticide was injected into or applied topically to adult houseflies, either alone or in the presence of the synergist. After a period the insects were homogenized and extracted, and the breakdown of the pyrethroid in the extract was examined. In all cases the pyrethroid had partly decomposed *in vivo*, this decomposition being inhibited by the synergist, less effectively with allethrin. Concurrently with this preliminary work, the same biosynthetically labelled pyrethroids have been independently used for studying their metabolism in the American cockroach, *Periplaneta americana* L. (Zeid, Dahm, Hein & McFarland, 1953) and in the housefly, *Musca domestica* L. (Earle, 1952). All the results are briefly compared and discussed below.

MATERIALS AND METHODS

Biosynthetic [^{14}C]*pyrethroids.* These were extracted in light petroleum from the heads of pyrethrum flowers allowed to mature in an atmosphere containing $^{14}CO_2$ by Pellegrini *et al.* (1952). The sample used in the present work consisted of a solution of 20 mg. of the 'purified' natural mixture of chrysanthemic and pyrethric esters dissolved in 20 ml. *n*-hexane, and was made available through the kindness of Dr A. C. Miller of the Gulf Research and Development Company, Pittsburgh, Pa. Reversed-phase paper chromatographic analysis (Fig. 3*a*) indicated on the basis of uniform ^{14}C-labelling that the mixture contained 41% of the chrysanthemic esters, 26% of the pyrethric esters and 33% unidentified impurities. After storing for 6 months at 0° the composition indicated was 50% of the chrysanthemic esters, 26% of the pyrethric esters and 24% unidentified impurities. The apparent increase in the chrysanthemic esters may have been due to decomposition in the earlier chromatograms. The specific activity of the crude mixture has been estimated to be 108 μc/g. (Pellegrini *et al.* 1952). For insect application a solution of either the chrysanthemic esters separated by reversed-phase paper chromatography or the unresolved mixture in A.R. acetone was used throughout.

Preparation of [^{14}C]*allethrin.* The labelled ester was prepared by the route indicated in Fig. 2. Asterisks denote the positions of isotopic carbon atoms. [1:3-$^{14}C_2$]Acetone (VII) (1·5 mc), purchased from the Radiochemical Centre, Amersham, Bucks, was diluted to 2·5 ml. with A.R. acetone. 3·8 g. purified selenium dioxide was dissolved in 20·0 ml. redistilled peroxide-free dioxan and 1·4 ml. water at 50° under a reflux condenser. The labelled acetone was added and the mixture gently refluxed for 4 hr. The mixture was distilled in nitrogen at atmospheric pressure. Water and dioxan were removed from the distillate under vacuum at 0°, when the labelled pyruvic aldehyde (VIII) remained as a clear syrup. The yield of aldehyde, determined volumetrically in rehearsal runs with unlabelled materials by the method of Friedemann (1927), was 40% based on the acetone. Ethyl 2-oxohex-5-ene-1-carboxylate (I) (2·27 ml.) was saponified with 9·3 ml. 10% KOH as described by Crombie *et al.* (1950) and the resulting acid (II) condensed with the total yield of (VIII) as described by the same authors. Provision was made here, however, for the quantitative recovery of CO_2 (IV), which proved to be unlabelled. The remaining stages—(V), (IX) and (X) to the final labelled ester (VI)—were conducted on about one-twenty-fifth of the original scale (Crombie *et al.* 1950) by using conventional high-vacuum microdistillation techniques. Oxidative losses during ether extractions were reduced by using peroxide-free ether containing 0·01% quinol. On the reduced scale yields tended to be slightly lower than those originally reported and fraction cutting of distillates was made less rigorous.

(±)-*trans*-Chrysanthemic acid (IX) was used for the acid chloride (X). The total yield of crude allethrin (VI) was 441 mg. This was purified as required by reversed-phase paper chromatography (see below), followed by elution of the allethrin zone in 95% ethanol at 5°. Radio-assay of paper-chromatograms (Fig. 3*b*) indicated that the crude ester contained 47% allethrin of specific activity approx. 57 μc/g. of pure ester. This specific activity was somewhat lower than that calculated on the basis of the labelled starting material. However, the labelled acetone had been stored for some months in a warm climate before dilution with inactive carrier and it seems likely that some decomposition had occurred.

The ester fraction was identical with (±)-*cis–trans*-allethrin when chromatographed by normal and reversed-phase techniques (see below) and was of comparable toxicity to the adult housefly when chromatographic eluates were bio-assayed by the technique of Hoskins & Messenger (1950). From the known stereochemistry of

allethrin the labelled product (VI) used in the present studies would be expected to be the racemic mixture (±)-allethronyl (±)-*trans*-chrysanthemate (Elliott, 1954). The preparation of the labelled ester was very greatly facilitated by the generous gifts of intermediates (I) and (IX) by Dr S. H. Harper. After a preliminary communication of this work had been published (Winteringham & Harrison, 1954), Acree, Roan & Babers (1954) also independently reported the synthesis of specifically labelled allethrin.

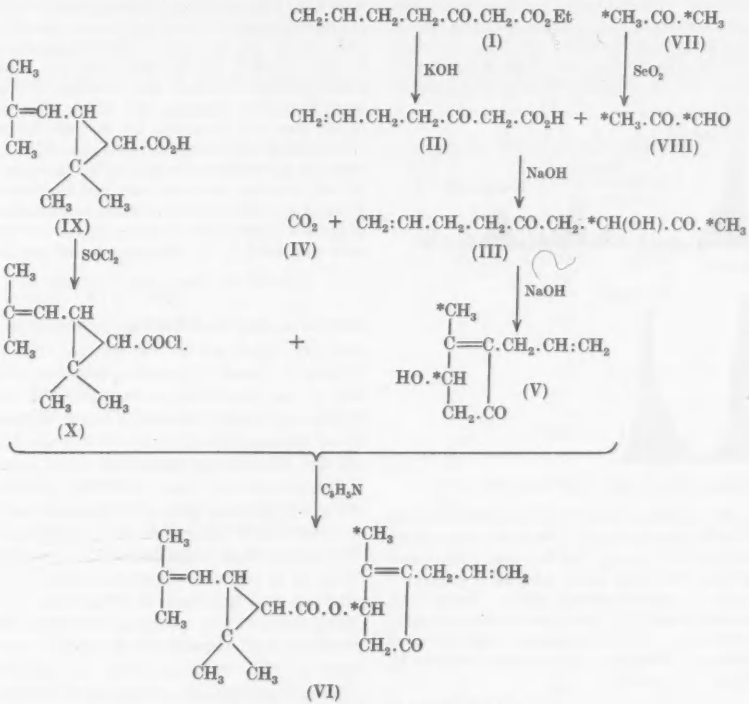

Fig. 2. Preparation of [^{14}C]allethrin.

Paper chromatography; radio-assay and bio-assay techniques. The chromatographic techniques described by Winteringham (1952*b*) were used with slight modifications. Confirmation of R_F values originally reported was made possible by the kind gifts of authentic samples of allethrin and cinerin I by Dr J. B. Moore and Dr M. S. Schechter respectively. Ascending chromatography was carried out in all-glass columns under reduced pressure (Winteringham, Bridges & Hellyer, 1955) at 25°. The original spraying technique for the location of separated fractions has been replaced by a simpler and equally sensitive dipping technique. After drying, the paper strip is dipped once in neutral 0·1% A.R. aqueous $KMnO_4$ and immediately washed free of excess of permanganate in distilled water. When almost dry the strip is dipped in a 0·5% solution (w/v) of A.R. benzidine in dry A.R. acetone containing 5% (v/v) glacial A.R. acetic acid. Pyrethroids and their unsaturated derivatives appear as intense blue zones. Less than 1 μg. of a separated pyrethroid is readily detected. Contamination of the strips (e.g. by fingering) is scrupulously avoided at all stages. Pyrethroids or their derivatives, whether or not recovered from insect tissue, were always applied to paper chromatograms in A.R. ether solution. Acetone, or some impurity in acetone, interfered with the separation of chrysanthemic acid from the alcohols derived from the pyrethroids in the simple two-stage technique described (Winteringham, 1952*b*). Labelled compounds were located and determined on the paper chromatograms by the methods described elsewhere (Winteringham, Harrison & Bridges, 1952). A sensitive 4π scanning technique was used for very low ^{14}C-activities (Harrison & Winteringham, 1955). In tissue samples ^{14}C was assayed as $Ca^{14}CO_3$ after wet oxidation of the sample (Winteringham, Harrison, Bridges & Bridges, 1955). For separating either the chrysanthemic esters of the biosynthetic sample or pure allethrin from the crude product (see above) it was found that up to 1 mg. of the mixture could be applied to a single 1·125 in. paper chromatogram without seriously interfering with the separation of the required esters. For the bio-assay of separated fractions by their toxicity to adult houseflies the fractions were eluted with 95% aqueous ethanol into the small vials used for

exposing the flies, the solvent was removed *in vacuo* and the residue bio-assayed by the method of Hoskins & Messenger (1950). Blank paper was similarly eluted for controls.

Treatment and extraction of insects. For studying the absorption and metabolism of the labelled pyrethroids *in vivo* the labelled pyrethroid, either alone or with piperonyl cyclonene, was dissolved in A.R. acetone and 1 μl. of this solution, which contained the amounts stated in Tables 1–4, was applied topically to the dorsal surface of the thorax of each of groups of ten to fifty 2- to 5-day-old adult female houseflies (*Musca domestica* L.) while under mild *cyclo*propane anaesthesia. Alternatively, the insecticide was injected intrathoracically in 1 μl. acetone. An Agla micrometer syringe (Burroughs Wellcome and Co., London) fitted with a 27 s.w.g. needle was used. Flies injected with 1 μl. acetone alone almost invariably survived and behaved

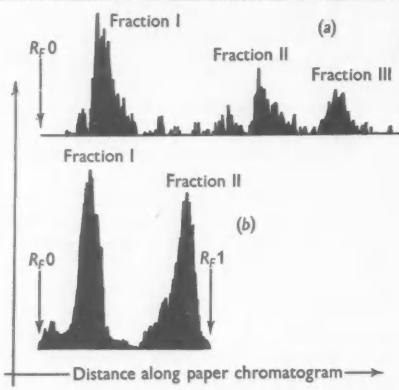

Fig. 3. Radiochromatograms of [^{14}C]pyrethroids. Tracings of original radiochromatograms. Peaks of radioactivity have been inked in for clarity. (*a*) Reversed-phase paper chromatography of natural esters labelled biosynthetically. Fraction I, chrysanthemic esters; fraction II, pyrethric esters; fraction III, non-insecticidal impurities. (*b*) Reversed-phase chromatographic separation of labelled allethrin. Fraction I, pure ester; fraction II, non-insecticidal impurities.

normally. The flies were transferred to a metabolism chamber of the type used for studying tissue phosphorus (Winteringham, Bridges & Hellyer, 1955), supplied freely with 5% aqueous glucose and observed for a 'metabolism' period of 24 hr. at 25° or 30°. Usually, up to one-half the number of treated insects remained alive during this period. Mortality was always greater in the presence of the synergist. In some cases provision was made for the collection of expired CO_2 during metabolism. This was precipitated as $CaCO_3$ and assayed for ^{14}C as described by Winteringham (1951). Some groups of flies were killed before treatment with the insecticide by immersion in water at 100° to inactivate tissue enzymes. At the end of the metabolism period all the treated flies, whether apparently dead or not, were briefly anaesthetized with *cyclo*propane. One group of ten flies was removed at random and rinsed with acetone. ^{14}C in this 'surface rinse' was determined and represented the unabsorbed pyrethroid. The rinsed flies were homogenized twice at room temperature with 2 ml. portions of ethanol, the homogenate was centrifuged, the clear combined supernatants were evaporated to dryness *in vacuo* and the residue obtained was dissolved in 1 ml. A.R. diethyl ether. Suitable portions of the ether extracts were then examined chromatographically. This enabled the relative proportions of the ^{14}C behaving as unchanged pyrethroids and as metabolites or decomposition products to be determined separately. These results are expressed as per cent total ^{14}C recovered in the extract. Various methods of extracting the absorbed pyrethroid and its decomposition products were tried [e.g. by diethyl ether extraction of acid homogenates by which 95% of a radioactive-bromine analogue of DDT could be recovered (Winteringham, Loveday & Harrison, 1951)], but the best recoveries (40–60% of the total pyrethroid applied) were obtained by simple ethanol extraction. The chemical fate of the unrecovered pyrethroid was not examined, but it could be recovered as $^{14}CO_2$ by wet oxidation of the tissues, and had not therefore been lost by expiration or excretion. In some experiments the surface rinse was also examined chromatographically. In other experiments the toxicities to houseflies of portions of the tissue extracts containing similar ^{14}C concentrations were compared by the bio-assay techniques mentioned.

RESULTS

All the results have been summarized in Tables 1–4 and are expressed to the nearest unit per cent in Tables 1, 2 and 4; greater precision was not justified by the statistical errors of the ^{14}C assays, these being a principal source of error throughout. The work was handicapped by the low specific activities of the labelled pyrethroids. For example, 1 μg. labelled allethrin was equivalent to 40 counts/min. on a background rate of 25 counts/min. in the 4π counter used (Harrison & Winteringham, 1955). Typically, 1 μg. allethrin or its metabolites was assayed as a chromatographed fraction spread over three 1 cm. sections of Whatman no. 1 paper. Each section was counted for 5 min. so that the coefficient of variation associated with the assay was ± 15%. Another cause of variation was the chemical instability of the pyrethroids under the conditions of extraction, concentration etc. of microgram quantities. Decomposition was likely to be greater during reversed-phase (R.P.) chromatography, which required 24 hr. or so, than the two-stage normal-phase (N.P.) technique, which could be completed within 1 hr. Moreover, the separation of 'non-pyrethroids' from pyrethroids was invariably more complete in the normal-phase technique. Only results obtained by the same chromatographic technique should therefore be compared. This has been done in Table 3, in which the net weight of absorbed or injected pyrethroid metabolized per fly has been obtained by subtracting the weight decomposed in the heat-killed flies from that in the live flies as estimated by the same chromatographic technique. It seems, however, that the results *in toto* are consistent.

Absorption. In all cases the absorption of topically applied pyrethroid was nearly complete in 24 hr. and was nearly double that of a bromine analogue of DDT under similar conditions (Winteringham, 1952*c*). The absorption was invariably reduced by the addition of piperonyl cyclonene (Table 1, Expts. 3*a* and 3*b*; Table 2, Expts. 3*a–d*, and 4*a–f*. See also Table 3). There was no evidence of selective absorption of the esters from an applied mixture (Table 1, Expts. 2 and 3).

Metabolism. All the results show an extensive decomposition of the applied or injected pyrethroid within 24 hr. This was invariably considerably less in flies which had been killed in hot water. The decomposition *in vivo* is believed therefore to be enzymic. Despite its greater chemical stability *in vitro*, allethrin was apparently metabolized *in vivo* to an extent comparable with that of the natural esters at a dose of about 1 μg.

The decomposition in the heat-killed flies was probably non-enzymic and occurred during application, extraction or exposure. A single result in Table 1, 2 (*b*) showed some anomalous decomposition of the esters in the heat-killed flies; not only were the results obtained by the two different chromatographic techniques in poor agreement but the decomposition was unusually high in all the extracts of this experiment. It seems likely that a

Table 1. *Metabolism of biosynthetic* [^{14}C]*pyrethroids by the adult housefly at* 25°

Expt.	Treatment	Metabolism time (hr.)	Pyrethroid absorbed (%)	^{14}C recovered (% of total)		Paper chromatographic technique*
				Unchanged pyrethroid	Non-pyrethroid	
1 (*a*)	1·5 μg. chrysanthemic esters applied topically	18	81	65	35	N.P.†
(*b*)	As in (*a*) but flies first killed in hot water	18	—	100	0	N.P.†
2 (*a*)	2·0 μg. natural mixture of esters applied topically	48	92	14 20	86 80	N.P.‡ R.P.‡
(*b*)	As in (*a*) but flies first killed in hot water	48	90	16 65	84 35	N.P.‡ R.P.‡
3 (*a*)	6·2 μg. natural mixture of esters applied topically	48	87	—	—	‡
(*b*)	6·2 μg. natural mixture + 10 μg. piperonyl cyclonene applied topically	48	79	—	—	‡
(*c*)	As in (*a*) but flies first killed in hot water	48	88	—	—	‡
4 (*a*)	0·7 μg. chrysanthemic esters injected intrathoracically	48	—	55	45	N.P.
(*b*)	As in (*a*) but flies first killed in hot water	48	—	100	0	N.P.
5 (*a*)	1·2 μg. chrysanthemic esters injected intrathoracically	48	—	69 40	31 60	N.P. R.P.
(*b*)	As in (*a*); duplicated expt.	48	—	74 57	26 43	N.P. R.P.
(*c*)	1·2 μg. chrysanthemic esters + 10 μg. piperonyl cyclonene injected intrathoracically	48	—	93 84	7 16	N.P. R.P.
(*d*)	As in (*a*) but flies first killed in water	48	—	92 87	8 13	N.P. R.P.
6 (*a*)	2·0 μg. chrysanthemic esters injected intrathoracically	24	—	50 46	50 54	N.P. R.P.
(*b*)	2·0 μg. chrysanthemic esters + 10 μg. piperonyl cyclonene injected intrathoracically	24	—	100	0	R.P.
(*c*)	As in (*a*) but flies first killed in hot water	24	—	95 87	5 13	N.P. R.P.

* R.P., reversed phase; N.P., normal phase in two stages (see text). These abbreviations are also used in Tables 2 and 3.
† Surface rinse examined by R.P. chromatography; no significant decomposition of chrysanthemic esters.
‡ All surface rinses examined by R.P. chromatography; no significant change in composition from that applied.

'control' extract was run as a 'live' extract in error in this experiment. In all cases piperonyl cyclonene appeared to inhibit the pyrethroid metabolism, but less effectively with allethrin. Unfortunately the synergist was injected with the natural esters and applied topically with allethrin. However, since most of the allethrin was absorbed, presumably with the synergist, the difference in inhibition was probably significant.

the point of application *A*. The reversed-phase technique will resolve the esters but not the hydrolysis products. Except in one or two doubtful cases the non-pyrethroid ^{14}C invariably behaved as an alcohol and remained at *A* (Fig. 4*b*). Allethrin, however, was labelled specifically in the alcohol portion of the molecule (Fig. 2), so that acid produced by hydrolysis would not be indicated by ^{14}C at *B*. This would not apply to the biosyn-

Table 2. *Metabolism of* [^{14}C]*allethrin by the adult housefly at* 30°

Expt.	Treatment	Metabolism time (hr.)	Pyrethroid absorbed (%)	^{14}C recovered (% of total)		Paper chromatographic technique
				Unchanged pyrethroid	Non-pyrethroid	
1 (*a*)	1 μg. allethrin applied topically	24	94	—	—	*
(*b*)	As in (*a*) but flies first killed in hot water	24	95	—	—	*
2 (*a*)	1 μg. allethrin applied topically	25	98	22 10	78 90	N.P.* R.P.*
(*b*)	As in (*a*) but flies first killed in hot water	25	82	76 92	24 8	N.P.* R.P.*
3 (*a*)	1 μg. allethrin applied topically	24	96	19	81	N.P.
(*b*)	1 μg. allethrin + 10 μg. piperonyl cyclonene applied topically	24	65	34	66	N.P.
(*c*)	As in (*a*) but flies first killed in hot water	24	95	65	35	N.P.
(*d*)	As in (*b*) but flies first killed in hot water	24	66	72	28	N.P.
4 (*a*)	1 μg. allethrin applied topically	24	98	18	82	N.P.
(*b*)	1 μg. allethrin + 5 μg. piperonyl cyclonene applied topically	24	88	40	60	N.P.
(*c*)	1 μg. allethrin + 10 μg. piperonyl cyclonene applied topically	24	82	53	47	N.P.
(*d*)	As in (*a*) but flies first killed in hot water	24	99	81	19	N.P.
(*e*)	As in (*b*) but flies first killed in hot water	24	93	72	28	N.P.
(*f*)	As in (*c*) but flies first killed in hot water	24	86	78	22	N.P.

* Expired CO_2 collected; no ^{14}C-activity detected.

Nature of the metabolites. The simple two-stage chromatographic technique (normal-phase as opposed to reversed-phase) proved to be the more useful technique for measuring the pyrethroid/non-pyrethroid ratio in the tissue extracts. In this technique (Winteringham, 1952*b*) the chromatogram is first developed for a few minutes with acid light petroleum (boiling range 40–60°), dried and then redeveloped for a longer period with ammoniacal light petroleum (boiling range 40–60°). Typical two-stage chromatograms are shown in Fig. 4*a* and *b*. The unchanged esters run with the ammoniacal solvent-front to *C* and free chrysanthemic acids with the acid solvent front to *B*, while allethronyl alcohol, for example, remains at

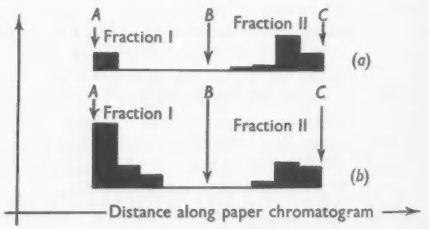

Fig. 4. Radiochromatograms of [^{14}C]pyrethroids. (*a*) Two-stage chromatography of allethrin etc. recovered from heat-inactivated insects. Fraction I, decomposition products; fraction II, unchanged ester. (*b*) As in (*a*) for live insects.

thetically labelled esters since they are labelled in both the alcohol and acid portions of the molecule, but there was no evidence of free chrysanthemic acid in ether extracts of acid homogenates, which are known to recover, partly at least, added unlabelled chrysanthemic acids. Radiochromatographic analysis of an alkaline hydrolysate of the biosynthetically labelled esters indicated, however, that the alcohol portion of the ester contained a larger proportion of ^{14}C than the acid portion of the molecule (cf. Earle, 1952). The evidence for the lack of free acids in the extracts is therefore only qualitative. Although little more than half the tissue ^{14}C was recovered in the ethanol extracts it is likely that the composition found was representative, the retention being due to some non-selective physical process. Recoveries in ethanol were incomplete from live or heat-inactivated tissues, and was apparently independent of the pyrethroid decomposition. This is illustrated in Table 4, which shows the ^{14}C-distribution in the fractions of Expt. 4, Table 2. It will be seen that 85–98 % of the ^{14}C applied as allethrin was accounted for in live and heat-inactivated tissues. This indicates

Table 3. *Net weights of tissue pyrethroids metabolized*

Weights have been estimated from the difference between the per cent of non-pyrethroid ^{14}C found in the extracts of live and of heat-killed flies respectively by the same chromatographic technique. It has been assumed that the non-pyrethroid/pyrethroid ratio found in the extract fully represents that which obtained in the tissues and that the absorption by heat-killed flies of topically applied pyrethroids is the same as that by live flies.

Expts.	Net wt. metabolized (μg.)	Chromatographic technique (see Tables 1 and 2)	Treatment of flies (see also Tables 1 and 2)
1 (*a*)–1 (*b*), Table 1	0·4	N.P.	1·5 μg. chrysanthemic esters applied topically
2 (*a*)–2 (*b*), Table 1	0	N.P.	2·0 μg. natural mixture of esters applied topically
	0·8	R.P.	
4 (*a*)–4 (*b*), Table 1	0·3	N.P.	0·7 μg. chrysanthemic esters injected
5 (*a*)–5 (*d*), Table 1	0·3	N.P.	1·2 μg. chrysanthemic esters injected
	0·6	R.P.	
5 (*b*)–5 (*d*), Table 1	0·2	N.P.	1·2 μg. chrysanthemic esters injected
	0·4	R.P.	
5 (*c*)–5 (*d*), Table 1	0	N.P.	1·2 μg. chrysanthemic esters + 10 μg. synergist injected
	0	R.P.	
6 (*a*)–6 (*c*), Table 1	0·9	N.P.	2·0 μg. chrysanthemic esters injected
	0·8	R.P.	
6 (*b*)–6 (*c*), Table 1	−0·3	R.P.	2·0 μg. chrysanthemic esters + 10 μg. synergist injected
2 (*a*)–2 (*b*), Table 2	0·5	N.P.	1·0 μg. allethrin applied topically
	0·8	R.P.	
3 (*a*)–3 (*c*), Table 2	0·4	N.P.	1·0 μg. allethrin applied topically
3 (*b*)–3 (*d*), Table 2	0·2	N.P.	1·0 μg. allethrin + 10 μg. synergist applied topically
4 (*a*)–4 (*d*), Table 2	0·6	N.P.	1·0 μg. allethrin applied topically
4 (*b*)–4 (*e*), Table 2	0·3	N.P.	1·0 μg. allethrin + 5·0 μg. synergist applied topically
4 (*c*)–4 (*f*), Table 2	0·2	N.P.	1·0 μg. allethrin + 10·0 μg. synergist applied topically

Table 4. *Recovery of ^{14}C topically applied to houseflies as labelled allethrin*

Fly group (see Table 2, Expt. 4)	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Allethrin applied (μg.)	1·00	1·00	1·00	1·00	1·00	1·00
Piperonyl cyclonene applied (μg.)	0	5·00	10·00	0	5·00	10·00
Initial condition of flies	Live	Live	Live	Dead	Dead	Dead
^{14}C in surface rinse (% ^{14}C applied)	2	10	17	2	6	14
^{14}C retained in tissue (% ^{14}C applied)	53	24	33	36	35	43
^{14}C extracted as allethrin (% ^{14}C applied)	8	21	22	48	38	32
^{14}C extracted as metabolites (% ^{14}C applied)	34	30	19	11	15	9
Total ^{14}C recovered (% ^{14}C applied)	97	85	91	97	94	98
Total ^{14}C extracted in ethanol (% ^{14}C applied)	42	51	41	59	53	41
Severity of symptoms (according to number of crosses) of flies exposed for 1 hr. to residue of evaporated ethanol extract (no symptoms in control group)	+	+ + +	+ + + +	+ +	+ + +	+ + + + +

that loss of ^{14}C by excretion or expiration did not occur or was relatively small. The absence of ^{14}C in expired carbon dioxide was confirmed in Expts. 1 and 2, Table 2, where it was estimated that labelled carbon dioxide equivalent to 3% or more of the applied ^{14}C would have been detected. In the experiments where the insecticide was applied topically it is likely that there were some losses by contact with the walls of the metabolism chamber, but these were not determined. The comparative toxicities to adult houseflies of portions of the ethanol extracts containing equal ^{14}C-activities are also shown in Table 4. The symptoms after 1 hr., i.e. number showing erratic behaviour, 'knock-down', etc., roughly paralleled the proportions of unchanged allethrin indicated chromatographically. Symptoms were always more severe in the presence of the synergist. Similar results were obtained in other experiments using male or female adult houseflies for the bio-assay. This strongly suggests that the metabolites were non-insecticidal and that the pyrethroid decomposition *in vivo* may be described as a detoxication mechanism.

DISCUSSION

In 24 hr. there was almost complete absorption by the adult housefly of topically applied pyrethroids, whether the separated chrysanthemic esters, a natural mixture of the chrysanthemic and pyrethric esters, or the fully synthetic (±)-*trans*-allethrin were applied. It was not surprising therefore that selective absorption of the chrysanthemic esters or pyrethric esters from a topically applied mixture had not occurred. Mammals are far more susceptible to the toxic effects of injected pyrethroids than to those applied percutaneously (Leonard, 1942; Carpenter, Weil, Pozzani & Smyth, 1950), which strongly suggests that the pyrethroids are not rapidly absorbed through the skin of mammals. Their relative contact toxicity to insects is therefore probably due to the relatively greater permeability of the insect integument and not to any particular biochemical susceptibility. There is substantial evidence that many of the halogenated-hydrocarbon contact insecticides similarly owe their selective action to the relative permeability of the insect integument to these substances (Winteringham & Barnes, 1955).

The absorption of topically applied pyrethroids was significantly reduced in the presence of piperonyl cyclonene (Tables 1 and 2). The effect occurred in live and heat-killed flies and was not therefore associated with pyrethroid metabolism which conceivably affected the concentration gradient across the integument. It was probably due to the dilution of the pyrethroid by the relatively large excess of synergist and the consequently smaller proportion of the total non-volatile material applied which the insect was capable of absorbing. The fraction of a labelled-bromine analogue of DDT absorbed by houseflies was similarly reduced at higher dosages (Winteringham, 1952*c*). Earle (1952) has also found that piperonyl butoxide retards the absorption of pyrethroids by houseflies.

Despite its greater chemical stability allethrin was apparently detoxified at 30° to about the same extent in 24 hr. as the natural esters at 25°. This increase in temperature increased the metabolism of a bromine analogue of DDT from 40 to 55% under the same conditions (Winteringham, 1952*c*). Inhibition of the detoxication of allethrin by piperonyl cyclonene was apparently less effective than that of the natural esters. This suggests that synergism of the natural esters is due to an inhibition of the natural detoxication mechanism *in vivo* and that the poorer effectiveness of piperonyl cyclonene as a synergist for allethrin is due to a less effective inhibition of the detoxifying enzyme system. It further suggests that the enzymes responsible for the detoxication of the natural and synthetic esters respectively are not identical. This leaves open the possibility of discovering a more effective allethrin synergist. This conclusion is not in agreement with that of Earle (1952), who compared the hydrolysis of a mixture of the natural esters with that of allethrin by roach lipase *in vitro* and concluded that the natural esters were hydrolysed more rapidly than allethrin. Hydrolysis was measured as unspecified acid formation. Apparent hydrolysis was equivalent to one-tenth or less of the added substrate, and this was less than the proportion of non-pyrethroids almost certainly present in his added substrates. On the basis of bio-assay techniques Earle also concluded that allethrin was more stable *in vivo* than the natural esters, and favoured this as an explanation of the better synergism between the natural esters and piperonyl butoxide.

Zeid *et al.* (1953) have studied the absorption of the biosynthetically labelled esters by the American cockroach, *Periplaneta americana*. These workers found ^{14}C in the carbon dioxide expired by roaches treated with the unfractionated mixture, and apparently assumed that it must have been derived from the 'radioactive insecticides'. The maximum fraction of applied ^{14}C recovered as expired carbon dioxide was 12%, which was less than the proportion of non-pyrethroid ^{14}C clearly present in their applied mixture. The labelled carbon dioxide could therefore have been derived entirely from the non-insecticidal impurities.

SUMMARY

1. A natural mixture of pyrethroids, labelled biosynthetically with ^{14}C, has been resolved by means of reversed-phase paper chromatography

into chrysanthemic esters, pyrethric esters and unidentified non-insecticidal impurities.

2. Allethrin, labelled with ^{14}C in the alcohol portion of the molecule, has been prepared on the millimole scale at a specific activity of about 60 μc/g. of pure esters and purified by means of reversed-phase paper chromatography.

3. Allethrin, the natural mixture of pyrethroids, or the chrysanthemic esters separated chromatographically was injected into or applied topically to adult houseflies. After a metabolism period the unchanged esters and their metabolites were extracted, resolved by paper chromatography and determined by radioactivity assay.

4. Significant and comparable fractions of all the applied pyrethroids were metabolized to relatively non-insecticidal substances within 24 hr. When the synergist piperonyl cyclonene was applied simultaneously with the pyrethroid, the metabolism was substantially inhibited, but least effectively in the case of allethrin. This suggests that the synergism involved an interference with the natural detoxication mechanism of the housefly and leaves open the possible development of a more effective allethrin synergist.

5. Absorption of the pyrethroids applied topically was almost complete within 24 hr. and was apparently non-selective from an applied mixture of esters. The presence of piperonyl cyclonene invariably retarded absorption in 24 hr., presumably by dilution of the pyrethroid on the insect integument.

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The Phosphorylation of Pantothenic Acid by *Lactobacillus arabinosus* 17–5

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It was shown previously (Pierpoint & Hughes, 1952, 1954) that during glycolysis, washed suspensions of *Lactobacillus arabinosus* 17–5 synthesize coenzyme A (CoA) from pantothenate and cystine, and that in the absence of cystine no CoA is synthesized, although the pantothenate is taken up and retained by the cells. The bound form of pantothenate can be extracted from the cells but is available as a growth-factor only after digestion with intestinal phosphatase. It was suggested therefore that, in the absence of cystine, pantothenate is phosphorylated. The present paper is concerned with further studies on the phosphorylation reaction and identification of the product as pantothenic acid 4′-phosphate. The formation of the 4′-phosphate is of especial interest because, although it forms a part of the CoA molecule (Baddiley & Thain, 1951*a*), it cannot be used by bacteria (King & Strong, 1951) or by animal tissues (King & Strong, 1950) for the synthesis of CoA. The significance of the phosphorylation is discussed in the light of recently suggested routes of CoA synthesis (Hughes, 1953; Novelli, 1953; Brown & Snell, 1953).

EXPERIMENTAL

Sources of phosphorylating enzyme. Dried preparations of *Lb. arabinosus* were prepared from cells that had been harvested after 16–18 hr. growth on the Nossal's (1951) medium and washed in 0·9% saline. They were thinly spread out on Petri dishes and dried over P_2O_5 in a desiccator continuously exhausted at 0·3 mm. Hg. When stored in the refrigerator, these preparations retained their phosphorylating activity for at least 3 months, though all activity was lost after 14 months at $-14°$.

Cell-free extracts were prepared by crushing washed cells, without abrasive, in the press described by Hughes (1951). If such extracts were not to be used immediately they were frozen and stored at $-15°$.

Measurement of enzyme activity. The ability of the various enzyme preparations to phosphorylate pantothenate was measured by following the disappearance of pantothenate from incubation mixtures as judged by microbiological assay with *Lb. arabinosus*. This was checked at frequent intervals by measuring the recovery of pantothenate after digestion with intestinal phosphatase. The method is based on the previous findings that phosphorylated pantothenates are inactive as growth factors for *Lb. arabinosus* but are hydrolysed to free pantothenate by the phosphatase (Pierpoint & Hughes, 1954). The standard conditions generally used were as follows. Samples of the material to be tested (0·1–0·5 ml.) were pipetted in $6 \times \frac{1}{2}$ in. test tubes containing pantothenate (4×10^{-4} M), ATP (adenosine triphosphate) (4×10^{-4} M), 0·1 M-Na–K phosphate buffer (pH 7·0), 0·1 ml. salts *B* (Barton-Wright, 1952) and 0·2 ml. NaF (0·2 M) in a total vol. of 2·0 ml. After mixing, the solutions were incubated at 37° and samples (0·2 ml.) taken at intervals depending upon the activity expected. The samples, in 10 ml. corked centrifuge tubes, were placed in a boiling-water bath for 10 min., diluted with 4 ml. water and centrifuged to remove protein. The pantothenate was estimated in the supernatant by microbiological assay (Pierpoint & Hughes, 1954).

Source and use of other enzymes. The intestinal phosphatase was a sample from Armour and Co. Ltd.; 0·1 ml. of a solution (10 mg./ml.) was added to 0·2 ml. of 0·1 M aminotrishydroxymethylmethane (tris) buffer (pH 8·0) and the sample of pantothenic acid phosphates containing between 0·4 and 1·2 μg. of bound pantothenic acid in 0·2 ml. water. After incubating for 3 hr. at 37°, the solution was diluted with 4 ml. of water, centrifuged if necessary, and the free pantothenic acid estimated in appropriate samples. The presence of 0·5 M fluoride in some of the samples of enzymically prepared pantothenate phosphate did not affect the action of the phosphatase.

Nucleotide phosphate estimation. ATP and ADP (adenosine diphosphate) were estimated by the method described by Krebs & Hems (1953). The adenosine derivatives were first separated from each other and from the other phosphate-containing compounds by two-way chromatography in *iso*propyl ether–formic acid, and *iso*butyric acid–ammonia–ethylenediaminetetraacetic acid (EDTA). The nucleotide-containing spots were detected by u.v. light and the phosphate spray of Hanes & Isherwood (1949). They were cut out, wet-ashed and analysed for phosphorus. If the solutions for estimation contained protein they were first heated on a boiling-water bath for 30 sec. The precipitated protein was removed by centrifuging and the estimation performed on the supernatants. This procedure did not affect the recoveries of ATP.

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Chromatography of pantothenic acid phosphates. This was performed on Whatman no. 1 filter paper which had been washed in EDTA (Eggleston & Hems, 1952). A 25 μl. sample containing total P at a concentration of about 10^{-3} M was put on paper in 5 μl. spots from an Agla micrometer syringe (Burroughs Wellcome and Co.) and dried by a current of cold air from a hair drier. The chromatograms were irrigated in a descending stream of either the *iso*butyric acid–ammonia–EDTA solvent of Krebs & Hems (1953) or the *n*-propanol–ammonia–water solvent of Hanes & Isherwood (1949). After 16–18 hr. the papers were removed and dried in a hot oven. The position of the phosphates was located by the acid molybdate spray of Hanes & Isherwood (1949).

Chemicals. The pantothenic acid phosphates used in this work had been prepared as described by Baddiley & Thain (1951*a*, *c*). With the exception of pantothenic acid 4′-phosphate, they were chromatographically homogeneous in both the solvents used. The 4′-phosphate contained a small amount (approx. 5%) of a faster moving phosphate-containing spot which was also observed by Baddiley & Thain (1951*c*). The ATP used was prepared by the method of LePage (1949). All the other chemicals used were commercial samples. Solution *B* (Barton-Wright, 1952) was used as a convenient source of inorganic ions.

RESULTS

Phosphorylation of pantothenic acid by cells of Lb. arabinosus 17–5

Vacuum-dried preparations of cells which had been grown in a pantothenic acid-deficient medium caused a disappearance of pantothenic acid when incubated in the presence of ATP, phosphate buffer (pH 7·1) and inorganic ions; no disappearance occurred when ATP was omitted from the mixture (Fig. 3). The pantothenic acid could be completely recovered by incubating the reaction mixture with intestinal phosphatase (Fig. 1). The same effect occurred with vacuum-dried preparations of cells grown on pantothenic acid-rich media. In this case, however, incubation with phosphatase resulted in recoveries of pantothenic acid in excess of that added (Fig. 2), indicating the initial presence of pantothenic acid phosphate derivatives in such cells.

Cell-free extracts of *Lb. arabinosus* prepared in a bacterial press (Hughes, 1951) also catalyse the phosphorylation. The reaction was further studied in dried cells, which could be more conveniently prepared. The enzyme system could not be extracted from the dried cells by leaving them for periods up to 3 days with water at 5°.

Effect of fluoride on the phosphorylation of pantothenic acid. Sodium fluoride (0·02 M) caused a 40% increase in the amount of pantothenic acid phosphorylated (Fig. 2). This is probably due to the inhibitory effect of this ion on the ATPases present in dried cells. Evidence in support of this was obtained by incubating cells with ATP, inorganic

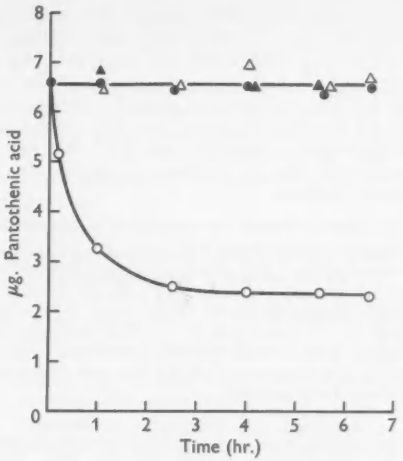


Fig. 1. The phosphorylation of pantothenic acid by dried pantothenate-deficient cells of *Lb. arabinosus*. Each tube contained 40 mg. dried cells, 0·02 ml. solution *B*, 0·5 ml. phosphate buffer (0·5 M, pH 7·1), pantothenic acid (7·3 μg.) and either water alone (●, ▲) or 1·0 ml. ATP (0·02 M), (○, △). Solutions were incubated at 37° and pantothenate was estimated before (●, ○) and after (▲, △) digestion with phosphatase.

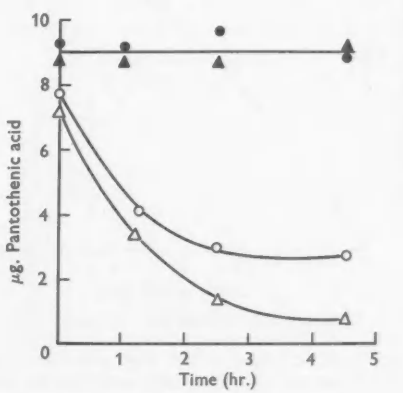


Fig. 2. Effect of fluoride on the phosphorylation of pantothenic acid by dried preparations of *Lb. arabinosus*. Cells were grown on excess of pantothenate. Dried cells (40 mg.) were incubated with 1·0 ml. ATP (0·05 M), pantothenic acid (7·3 μg.), 0·02 ml. solution *B*, 0·5 ml. of phosphate buffer (0·5 M, pH 7·0) and either water to 3·35 ml. (○, ●) or 0·35 ml. NaF (0·2 M) and water to 3·35 ml. (△, ▲). Pantothenate was estimated before (○, △) and after (●, ▲) digestion with phosphatase.

ions and phosphate buffer, in either the presence or absence of fluoride, and adding pantothenic acid 4·5 hr. later. Phosphorylation occurred only in those solutions that contained fluoride. The dried cells did not catalyse the phosphorylation when both ATP and pantothenic acid were added after the incubation, suggesting that ATP, but not the fluoride ion, has a 'protective' action on the enzyme involved.

Table 1. *Effect of pantothenic acid concentration on the amount of phosphorylation*

Tubes containing 20 mg. dried cells, 0·5 ml. ATP (0·02 M), 0·1 ml. solution *B*, 0·2 ml. NaF (0·2 M), 0·25 ml. phosphate buffer (0·5 M, pH 7·1), pantothenic acid and water to 1·7 ml. were incubated for 6 hr. and pantothenate was assayed as described.

Concn. of pantothenic acid (μM)	Amount phosphorylated (μg.)	Amount phosphorylated (%)
18	6·1	83
88	32·3	98
440	47·0	28
880	74·0	22

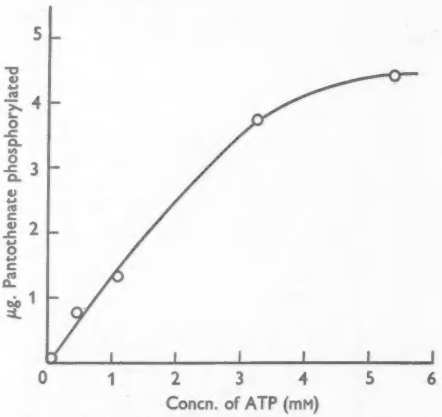


Fig. 3. Variation of phosphorylation of pantothenic acid with ATP concentration. Dried cells (40 mg.) were incubated for 6·5 hr. at 37° with pantothenate and various amounts of ATP and the remaining free pantothenate was assayed.

Effect of concentrations of ATP and pantothenic acid on the phosphorylation. Increasing the concentration of pantothenic acid from $1{\cdot}8 \times 10^{-5}$ M to $8{\cdot}8 \times 10^{-4}$ M increased the amount of phosphorylation that occurred in the presence of NaF (Table 1). The concentration of ATP used in early experiments was approximately optimal for the conditions under which it was used (Fig. 3). In most subsequent experiments on phosphorylation, the concentrations of pantothenic acid and ATP used were 4×10^{-4} M. Under these conditions the ATP did not limit the reaction, and the large proportion of added pantothenic acid that was phosphorylated allowed its disappearance to be measured accurately.

Omitting inorganic Mg, Mn and Fe ions from the system reduced the amount of phosphorylation by 20%. Addition of cysteine or cystine in quantities up to 10 times the equivalent amount of pantothenic acid did not affect the amount of phosphorylation or the recovery of pantothenic acid after phosphatase digestion. This is in contrast to the reaction in intact deficient cells, where the addition of cysteine reduces the amount of pantothenate recovered by digestion with phosphatase because of the synthesis of CoA (Pierpoint & Hughes, 1954).

Product of the phosphorylation reaction

The enzymic phosphorylation of pantothenic acid may result in the formation of one of five possible compounds. These are pantothenic acid 2′-phosphate, pantothenic acid 4′-phosphate, pantothenic acid 2′:4′-cyclic phosphate, pantothenic acid 2′:4′-diphosphate and the acyl phosphate pantothenoyl phosphate (Fig. 4). The following comparison of the properties of the enzymically produced material with samples of the synthetically prepared monophosphates of pantothenic acid supports the conclusion that the 4′-phosphate is formed by the enzyme.

Pantothenic acid

$HO.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO_2H$

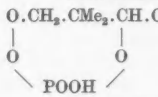
Pantothenic acid 2′-phosphate

$HO.CH_2.CMe_2.CH(O.PO_3H_2).CO.NH.CH_2.CH_2.CO_2H$

Pantothenic acid 4′-phosphate

$H_2O_3P.O.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO_2H$

Pantothenic acid 2′:4-cyclic phosphate



Pantothenic acid 2′:4′-diphosphate

$H_2O_3P.O.CH_2.CMe_2.CH(O.PO_3H_2).CO.NH.CH_2.CH_2.CO_2H$

Pantothenoyl phosphate

$HO.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO.OPO_3H_2$

Fig. 4. Phosphates of pantothenic acid.

(1) *Action of phosphatase on pantothenic acid phosphates.* King & Strong (1951) and Baddiley & Thain (1951*a*) report that the phosphates listed above, with the exception of the 2′:4′-cyclic compound, are hydrolysed by intestinal phosphatase. Table 2 shows the effect of intestinal phosphatase on synthetic phosphates under conditions in which 90–100 % of the enzymically produced phosphate is converted into pantothenic acid. Although none of the compounds was completely hydrolysed, D(+)-pantothenic acid 4′-phosphate was hydrolysed to a major extent (60–75 %). Increasing the time of incubation did not increase the degree of hydrolysis, but apparently decreased it (Table 3) owing to the instability of pantothenic acid at pH 8·3. The low results obtained with the racemic compounds are due to the fact that the pantothenic acid assay is specific for the D(+)-form.

(2) *Stability of pantothenic acid phosphates.* The phosphates of pantothenic acid are more stable than pantothenic acid in acid or alkaline solution at 100° (Baddiley & Thain, 1951*b*), and measurement of the rate of hydrolysis thus affords a method of distinguishing between 2′- and 4′-phosphates. In alkaline solution (0·5 N-NaOH) the phosphate groups of both compounds are stable, while the amide linkage in the 2′-phosphate is more labile than that of the 4′-derivative. Fig. 5 shows the results of comparing the stability of the enzymically produced phosphate with a sample of synthetic pantothenic acid 4′-phosphate. The behaviour of the two compounds is very similar, and the stability towards alkali differentiates both from the 2′-phosphate.

Lack of any strictly comparable data on the stability of the cyclic 2′:4′-monophosphate and the 2′:4′-diphosphate permits no further conclusions to be drawn. In alkaline solutions, at least, the latter

Table 2. *Effect of intestinal phosphatase on pantothenic acid phosphates*

The various phosphates in 0·2 ml. tris buffer (pH 8·3) were incubated for 3 hr. at 37° with intestinal phosphatase solution, approx. 5 units (Schmidt & Thannhauser, 1943). Free pantothenate was assayed as described.

Expt.	Substrate	Amount added (μg. bound pantothenic acid)	Pantothenic acid liberated (%)
1	D(+)-Pantothenic acid 4′-phosphate	0·88	60
	DL-Pantothenic acid 4′-phosphate	0·88	30
	Enzymically prepared phosphate	0·39	92
2	D(+)-Pantothenic acid 4′-phosphate	0·44	60
	DL-Pantothenic acid 4′-phosphate	0·44	28
	D(+)-Pantothenic acid 2′-phosphate	0·44	11
	DL-Pantothenic acid 2′-phosphate	0·44	6·0
	D(+)-Pantothenic acid 2′:4′-phosphate	2·5	0·4
3	D(+)-Pantothenic acid 4′-phosphate	0·44	74
	D(+)-Pantothenic acid 2′-phosphate	0·44	18
	D(+)-Pantothenic acid 2′:4′-phosphate	1·25	0
	D(+)-Pantothenic acid 2′:4′-phosphate	0·62	0

Table 3. *Time course of phosphatase action on pantothenic acid 4′-phosphate*

Either D- or DL-pantothenic acid 4′-phosphate (10^{-5} M) was incubated with phosphatase and the free pantothenic acid liberated and assayed as described.

Substrate	Pantothenic acid liberated (%) 1·5 hr.	3 hr.	6·5 hr.	19·5 hr.
D-Pantothenic acid 4′-phosphate	56	52	52	26
DL-Pantothenic acid 4′-phosphate	26	27	26	8·5

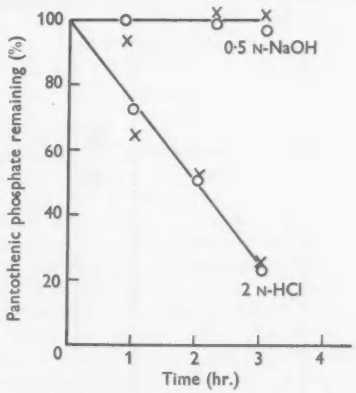


Fig. 5. Stability of pantothenic acid phosphates. 0·5 ml. 2·5 N-NaOH or 0·5 ml. of 10 N-HCl was added to 0·196 ml. of 10^{-5} M synthetic DL-pantothenic acid 4′-phosphate (×), or 0·174 ml. enzymically produced (○) phosphate (10^{-5} M with respect to bound pantothenic acid). The volumes were adjusted to 0·25 ml. with water, and the tubes placed in a boiling-water bath for various times. The amounts remaining undecomposed were measured by assaying pantothenic acid released by phosphatase.

compound would be expected to behave like the 4′-phosphate, into which it is rapidly converted under these conditions (Baddiley & Thain, 1951*c*).

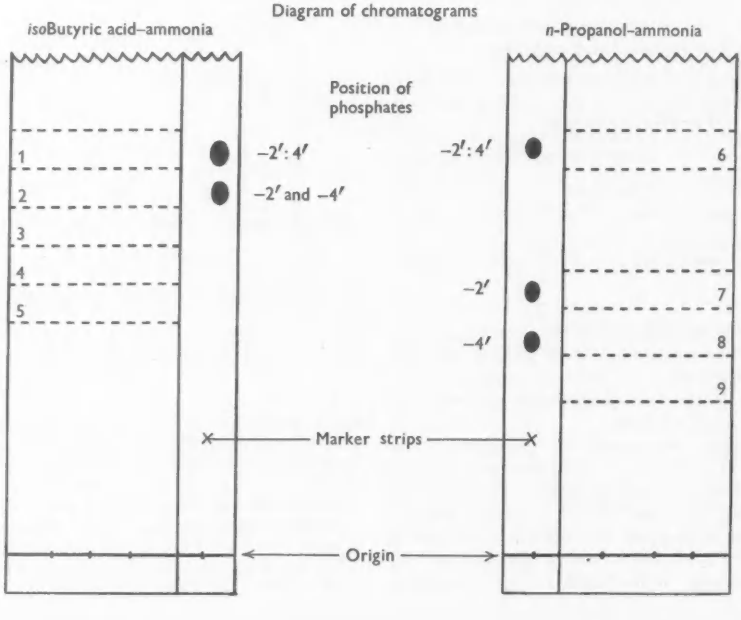
(3) *Chromatography of pantothenic acid phosphates.* The solution in which pantothenic acid was phosphorylated contained large amounts of other materials, which were removed as follows. The reaction mixture was heated on a boiling-water bath for 4 min. and the precipitated protein removed by centrifugation. Barium hydroxide (0·3N) was added and insoluble barium phosphates were removed by centrifuging. The solution was then heated at 100° for 5 min. to destroy the remaining pantothenic acid. The solution was adjusted to pH 6 with sulphuric acid; after barium sulphate had been removed by centrifuging, the

Table 4. *Chromatographic identification of the phosphorylated pantothenic acid*

Cells (100 mg. dry wt.) were incubated overnight with the following solutions: 4·5 ml. ATP (0·02M), 2·2 ml. phosphate buffer (0·5M), 1·2 ml. NaF (0·2M), 0·07 ml. solution *B*, 0·6 ml. pantothenic acid (10^{-4}M). The pantothenic acid phosphate was concentrated as described in the text and chromatographed for 16 hr. The positions of synthetic phosphates were detected on the chromatograms and the areas indicated in the diagram cut out and eluted with water. The eluates were assayed for pantothenic acid before and after digestion with alkaline phosphatase.

Expt.	Final solvent	Area on chromatogram	Corresponding phosphate	Pantothenic acid liberated by phosphatase (μg.)	Proportion of total pantothenic acid recovered (%)
I	*iso*Butyric acid–ammonia	1	2′:4′-cyclic	0·03	12
		2	2′- and 4′-	0·26	88
		3	—	0	0
		4	—	0	0
		5	—	0	0
II	*n*-Propanol–ammonia	6	2′:4′-cyclic	0	0
		7	2′-	0	0
		8	4′-	0·12	97
		9	—	0·004	3
III	*n*-Propanol–ammonia	6	2′:4′-cyclic	0	0
		7	2′-	0·044	18
		8	4′-	0·196	80
		9	—	0·004	2

Diagram of chromatograms



solution was passed through a column of Amberlite IR-120 resin to remove cations, and was then freeze-dried. For chromatography, the solid was dissolved in a little water and neutralized with ammonia. Chromatography of the product showed that it contained a number of phosphates, some of which absorbed in the u.v. region (Baddiley & Mathias, 1954), and that the enzymically formed pantothenic acid phosphate was spread over a wide range (2–3 in.) of the chromatogram. Consequently, the material was further purified by chromatographic separation in *iso*butyric acid–ammonia–EDTA (Krebs & Hems, 1953), and elution of those areas of the chromatogram that contained the phosphate-bound pantothenic acid. When the eluted material was run again in the *iso*butyric acid–ammonia–EDTA solvent it behaved like either the 2′- or the 4′-phosphate. (These cannot be distinguished in this solvent.) In *n*-propanol–ammonia–water it behaved like the 4′-phosphate (Table 4). No synthetic 2′:4′-diphosphate was available to use as a marker spot in these studies, but it has been shown (Baddiley & Thain, 1951*b*) to move much more slowly than any of the other pantothenic acid phosphates in both the above solvents. Its R_F value in *n*-propanol–ammonia–water was 0·13, compared with 0·45 for the 2′-phosphate and 0·33 for the 4′-phosphate. The corresponding values in *iso*butyric acid–ammonia–water were 0·18, 0·4 and 0·37.

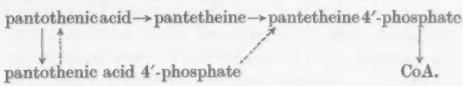
DISCUSSION

The evidence presented makes it reasonably certain that the enzyme system which has been studied catalyses the formation of D(+)-pantothenic acid 4′-phosphate. Although this compound has not been isolated in quantities sufficient for the application of conventional chemical analysis, it has been shown that its stability in acid and alkali, and its chromatographic behaviour, correspond with those of a synthetic sample of this material. There is some discrepancy in the behaviour of the synthetic phosphate and the enzymically produced material, in that only 75% of the pantothenic acid content of the former is liberated by intestinal phosphatase under conditions where all (90–100%) of the pantothenic acid of enzymically formed material was released. This discrepancy may be due to partial racemization of the D(+)-pantothenic acid during the synthesis. King & Strong (1950) and Baddiley & Thain (1951*a*) report that 90–100% of the pantothenic acid of both 2′- and 4′-phosphates could be liberated by the action of intestinal phosphatase. The conditions used by the former workers were slightly different from those used in the present work.

Although ADP was formed during the phosphorylation of pantothenate by dried cell preparations, the amounts formed were very much larger than expected. This is due to the presence in the preparation of ATPases which are only partly inhibited by fluoride. It is not certain therefore whether ATP donates phosphate to pantothenate directly.

Phosphorylation of pantothenic acid and CoA synthesis. Hoagland & Novelli (1954) have shown that in liver CoA can be synthesized from pantetheine in three steps: (1) pantetheine + ATP → pantetheine 4′-phosphate + ADP; (2) pantetheine 4′-phosphate + ATP → dephospho-CoA + pyrophosphate; (3) dephospho-CoA + ATP → CoA + ADP.

These authors did not test the preparation which phosphorylates pantetheine for its ability to phosphorylate pantothenate. Since pantothenic acid 4′-phosphate is utilized neither by liver for CoA synthesis (King & Strong, 1951) nor as a growth factor for bacteria, it is now generally assumed that in the synthesis of CoA the phosphate is attached to 4′-carbon only after pantothenate has been converted into pantetheine. The present observations do not rule out the possibility that pantothenic acid 4′-phosphate is an intermediate in CoA synthesis in bacteria. Its inactivity with intact cells may be due to the inability of certain phosphoric esters to enter the cell. Since the formation of pantothenic acid 4′-phosphate has been detected only in the absence of cystine, it seems feasible that it might serve as a storage form of pantothenate. The two alternative roles are shown in the following scheme:



SUMMARY

1. Dried cells of *Lb. arabinosus* catalyse the disappearance of pantothenic acid when incubated with ATP and phosphate buffer.

2. Phosphatase treatment results in a complete recovery of the pantothenic acid, indicating that the substance is being converted into a phosphoric ester. Sodium fluoride increases the extent of phosphorylation, probably owing to its inhibition of ATP-splitting enzymes present in dried cells.

3. The pantothenic acid derivative that is formed has been concentrated, and its properties have been compared with those of the synthetic pantothenic acid monophosphates. Its hydrolysis by intestinal phosphatase, stability to acid and alkali, and chromatographic properties suggest that it is pantothenic acid 4′-phosphate.

We wish to thank Professor H. A. Krebs, F.R.S., for his interest in this work and Mr M. Clegg and Miss H. Marsden for technical assistance. The work was aided by a grant from the Rockefeller Foundation.

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Studies on Sulphatase

11. THE PURIFICATION AND PROPERTIES OF THE ARYLSULPHATASE OF *ALCALIGENES METALCALIGENES**

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A strain of *Alcaligenes metalcaligenes*, which was isolated from an inter-tidal mud, possessed considerable arylsulphatase activity, and a preliminary purification of the enzyme has been made (Dodgson, Melville, Spencer & Williams, 1954). The present work describes the further purification of the arylsulphatase and, from consideration of its properties, gives some indication of its possible functional groups.

MATERIALS AND METHODS

Arylsulphatase substrates. The potassium salts of *p*-nitrophenyl (NPS), *p*-acetylphenyl (APS) and nitrocatechol (NCS) sulphates were prepared by the method described previously (Dodgson *et al.* 1954).

Other substrates. Potassium chondroitin sulphate was prepared from tracheal cartilage by a modification of the method of Einbinder & Schubert (1950). The potassium salts of *p*-nitrophenyl phosphate and phenyl acetate were commercial samples.

Determination of arylsulphatase activity. Enzyme activity was determined by the method previously described (Dodgson *et al.* 1954) and was expressed in terms of nitrogen, as determined by the micro-Kjeldahl method of Markham (1942), or protein, as determined by the method of Lowry, Roseborough, Farr & Randall (1951).

Determination of nucleic acid. Ribonucleic and deoxyribonucleic acids were estimated by the method of Schmidt & Thannhauser (1945). The ratio of the spectrophotometric readings at 280 and 260 mμ. was used to locate nucleic acid in the electrophoresis experiments (Warburg & Christian, 1941).

* Part 10: Dodgson, Rose & Spencer (1955).

EXPERIMENTAL AND RESULTS

Preparation of enzyme concentrate

The enzyme was concentrated to stage *B* as previously described (Dodgson *et al.* 1954). Attempts to purify the enzyme further by ammonium sulphate fractionation and adsorption techniques with ion-exchange resins, alumina, calcium carbonate and benzoic acid were of little value since losses in total enzyme activity were not compensated by the degree of purification achieved. However, paper electrophoresis of the stage *B* enzyme suggested that considerable purification was possible by the use of this technique.

Electrophoresis experiments. The enzyme solution (0·1 ml. of a solution of a 0·1 g. stage *B* in 0·6 ml. of 0·1 M phosphate buffer, pH 8·0) was run for 18 hr. on vertically held Whatman no. 100 filter paper at 110 v in the presence of 0·1 M phosphate, pH 8·0. The paper was dried at room temperature and cut into 1 cm. strips. Protein was located by staining with bromophenol blue (see Flynn & de Mayo 1951) and arylsulphatase activity was located, according to the method described by Dodgson, Spencer & Thomas (1955), by incubating each strip with 0·5 ml. of 0·0015 M NPS in 0·1 M phosphate at pH 8·75.

The enzyme migrated towards the anode and was the fastest-moving protein component. In subsequent experiments the enzyme was eluted from the relevant portion of the paper with water.

Table 1. *Stages in the purification of the arylsulphatase of* Alcaligenes metalcaligenes

Enzyme activity was measured against 0·0015M NPS in 0·1M phosphate buffer, pH 8·75 at 37·5° for 1 hr. One NPS unit equals 1 μg. *p*-nitrophenol liberated under these conditions.

	Enzyme activity in NPS units		
	per mg. N	per mg. RNA phosphorus	Enzyme activity remaining (%)
Whole cells	53	—	100
Stage *A*	370	26 000	80
Stage *B*	1 190	25 400	50
Electrophoretically purified enzyme	16 500	34 300	35

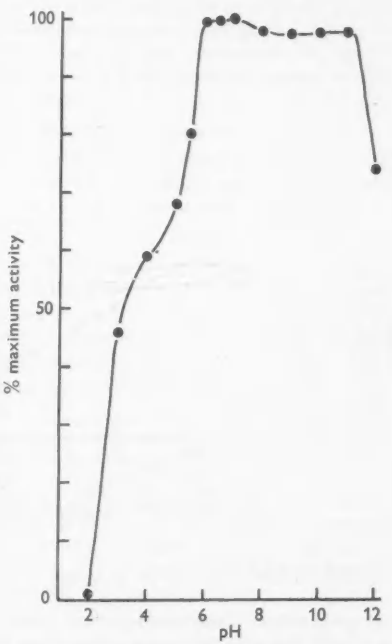


Fig. 1. Inactivation of the arylsulphatase of *A. metalcaligenes* with pH. The enzyme solution was adjusted to the required pH with HCl or NaOH and allowed to stand at 0° for 45 min. An equal volume of 0·2M phosphate buffer pH 8·75 was then added, the pH of the mixture checked to pH 8·75, and the arylsulphatase activity determined by the use of NPS under the optimum conditions.

Spectrophotometric examination of the eluate revealed a peak of absorption at 260 mμ., a region where nucleic acid absorbs strongly. Estimation of the organically bound phosphate present in the eluate by the method of Schmidt & Thannhauser (1945) also indicated the presence of nucleic acid. The results given in Table 1 suggested a correlation between enzyme activity at various stages of purification and the concentration of ribonucleic acid phosphorus. A similar correlation was also noted during the preliminary attempts to purify the enzyme by ammonium sulphate fractionation or by adsorption techniques. It was subsequently noted that the curve showing the enzyme activity remaining after allowing the enzyme to stand at various pH values for 45 min. before assay (Fig. 1) was substantially the same as the curve showing the effect on pH on the degree of polymerization of deoxyribonucleic acid (Gulland, Jordan & Threlfall, 1947). A particular point of comparison was the sharp drop in viscosity of deoxyribonucleic acid and activity of the enzyme which occurred below pH 6 and above pH 11.

Binkley (1952, 1954) has indicated that the peptidase activities of a cell are a property of the polynucleotides of the cell. In view of the experimental results obtained in the present work the possibility that polynucleotides were also responsible for arylsulphatase activity had to be considered. The fact that incubation with crystalline ribonuclease had no effect on the arylsulphatase activity did not necessarily rule it out, but the rapid inactivation of the arylsulphatase by trypsin showed that the enzyme was essentially protein in nature (see Binkley, 1954). However, the possibility that protein associated with nucleic acid was responsible for arylsulphatase activity could not be excluded at this stage.

Separation of the nucleic acid and the arylsulphatase. When the paper-electrophoresis runs were prolonged beyond 18 hr. there was some indication of separation of nucleic acid and enzyme (Fig. 2). Subsequently the portion of the paper containing the bulk of the arylsulphatase activity was cut out, the enzyme eluted with water and the eluate was concentrated by freeze-drying. Paper electrophoresis of the concentrated solution resulted in a still greater degree of separation (Fig. 2), and arylsulphatase, free from nucleic acid, could be eluted from the paper. The apparent association of nucleic acid and arylsulphatase activity observed in the preliminary experiments must therefore be regarded as fortuitous.

Electrophoretic concentration of the enzyme. A 17% (w/v) solution of the stage *B* powder in water was run on Whatman no. 100 paper during 24 hr. under the conditions described previously. Several papers, each carrying 0·1 ml. of the enzyme

solution, were run simultaneously and the enzyme and nucleic acid were subsequently located on one of the papers. The other papers were cut so as to retain the bulk of the arylsulphatase activity with a minimum contamination from nucleic acid, suspended in water and dialysed overnight. The

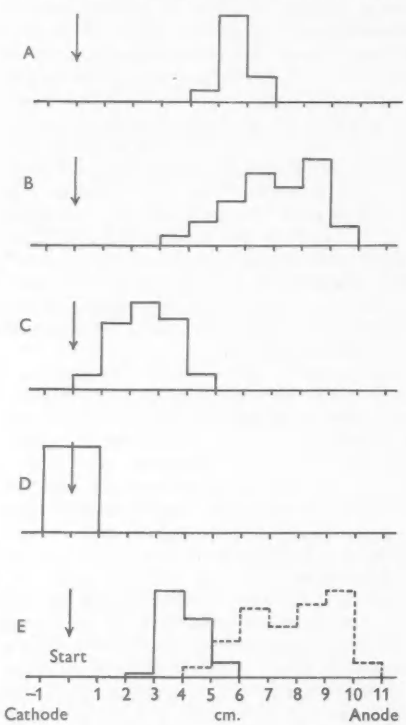


Fig. 2. Paper electrophoresis. Stage *B* concentrate was run on vertically held Whatman no. 100 paper in 0·1M phosphate buffer pH 8·0 for 24 hr. at 110v and the positions of the arylsulphatase (A), nucleic acid (B), esterase (C) and phosphatase (D) were determined by the method described in the text. A better separation (E) of nucleic acid (interrupted line) and arylsulphatase (solid line) was achieved by re-running, under the same conditions, a concentrate of the arylsulphatase eluted from the sixth cm. of A (see text). The distribution patterns shown have been drawn with an arbitary scale.

filtered solution was freeze-dried to give a colourless powder which was stable at 0°. One g. of the powder liberated 1·480 g. *p*-nitrophenol from 0·0015M NPS in 0·1M phosphate, pH 8·75 at 37° over 1 hr. On a nitrogen basis this represents a 300-fold purification of the enzyme with a retention of 35% of the original activity (Table 1). Subsequent experiments were performed with enzyme concentrates prepared in this way.

Presence of other enzymes in the concentrate. An esterase, active towards phenyl acetate, and a phosphatase, active towards *p*-nitrophenyl phosphate, were present at stage *B*. The behaviour of the esterase during electrophoresis was followed by incubating 1 cm. strips of the paper with a saturated solution of phenyl acetate in 0·1M phosphate, pH 8·75, and subsequently treating according to the directions of Aldridge (1954). Phosphatase was located by incubating the strips with 0·01M disodium *p*-nitrophenyl phosphate in 0·1M phosphate, pH 8·75, and subsequent detection of liberated *p*-nitrophenol. The two enzymes migrated towards the anode at much slower rates than the arylsulphatase and were thus eliminated during the purification procedure by cutting the papers as described (Fig. 2).

The electrophoretically purified enzyme was free from chondrosulphatase since it did not liberate inorganic sulphate from a solution (0·75%, w/v) of potassium chondroitin sulphate in 0·5M acetate at pH's 7 and 8. It has been previously reported that the stage *B* concentrate contains no glycosulphatase or glucuronidase activity (Dodgson *et al* 1954).

Properties of the enzyme concentrate

The enzyme concentrate gave pH/activity and substrate concentration/activity curves which were very similar to those obtained previously with

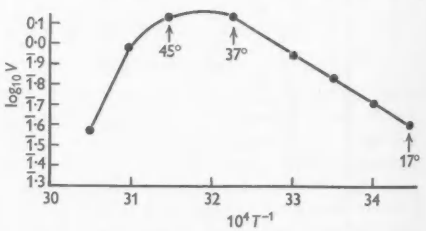


Fig. 3. The effect of temperature on the activity of arylsulphatase against NPS. The results are plotted in the form $1/T$ (° K) against $\log_{10} V$, for calculation of the activation energy of the enzyme.

the stage *B* enzyme (see Dodgson *et al.* 1954). The ratio of the observed activities towards the three substrates under the respective optimum conditions was 5·2 (APS):5·0 (NPS):1·0 (NCS) calculated on a molar basis; when account was taken of the inhibition by excess of substrate and the true $V_{max.}$ calculated (Lineweaver & Burk, 1934, case III), the ratio became 15·1:14·4:1·0. The Michaelis constants, K_m, for the three substrates, calculated

by the method of Lineweaver & Burk were $9{\cdot}0 \times 10^{-4}$ M (APS), $4{\cdot}75 \times 10^{-4}$ M (NPS) and $2{\cdot}2 \times 10^{-4}$ M (NCS), showing that the affinity of the enzyme for substrate ($1/K_m$) was greatest with NCS.

Table 2. *The effect of inhibitors on the arylsulphatase of* Alcaligenes metalcaligenes

Inhibitors were allowed to act as described in the text and the residual arylsulphatase activity was measured for 1 hr. at 37° against NPS under the optimum conditions.

Inhibitor	Concentration of inhibitor M	% inhibition
NaCl	0·025	0
NaF	0·025	0
Na_2SO_4	0·025	0
$NaHSO_3$	0·025	17
NaCN	0·025	82
	0·0125	69
	0·0063	51
Na_2SO_3	0·025	34
	0·0125	28
	0·0063	19
$BaCl_2$	0·025	100
Urea	0·025	0
Glucose 6-sulphate	0·025	17
Potassium ethyl sulphate	0·025	12
Sodium benzenesulphonate	0·025	33
Phenylhydrazine	0·0001	87
	0·00005	73
	0·000025	52
Hydrazine	0·025	43
Semicarbazide	0·025	21
Thiosemicarbazide	0·025	33
Dimedone	0·5% (w/v)	27
Girards' reagent P	2·5% (w/v)	25
Hydroxylamine	0·025	97
Phosphate* (pH 6·3 and 8·75)	0·1	0

* The activity in the presence of phosphate was compared against control determinations in which enzyme and substrate, previously adjusted to the appropriate pH with NaOH or HCl, were incubated together in the absence of phosphate buffer. The pH of these controls did not vary during the course of the experiment.

The heat of activation was determined by measuring the activity of the enzyme at various temperatures against NPS under the optimum conditions and using a 30 min. incubation period. The plot of $1/T$ (in °K) against $\log_{10} V$ (Fig. 3) was a straight line over the range 17–37° and gave a heat of activation (Arrhenius, 1889) of 11 kcal./mole. This value should be compared with that for ox-liver sulphatase A, 14 kcal./mole at high enzyme concentrations (Roy, 1953), ox-liver sulphatase B, 13 kcal./mole (Roy, 1954), and the arylsulphatase of *Charonia lampas*, 28 kcal./mole (Soda & Yoshida, 1948).

The effect of inhibitors. A number of compounds which were known to inhibit arylsulphatases from other sources were tested against the *Alcaligenes* enzyme. The inhibitor (0·3 ml.) in 0·1 M phosphate buffer adjusted to pH 8·75 was mixed with 0·3 ml. enzyme solution and the mixture kept at 37° for 15 min. before the addition of 0·6 ml. 0·0015 M NPS in 0·1 M phosphate, pH 8·75. After incubation for 1 hr. the liberated *p*-nitrophenol was estimated in the usual way. The results are listed in Table 2. The *Alcaligenes* enzyme was inhibited by aldehyde reagents such as cyanide, phenylhydrazine and hydroxylamine. In this respect it resembles the arylsulphatases of clarase (Dzialoszynski, 1950), Takadiastase (Robinson, Smith, Spencer & Williams, 1952) and the locust (Robinson, Smith & Williams, 1953). In contrast, the enzyme of the limpet (Dodgson & Spencer, unpublished results) and the soluble enzymes of rabbit (Maengwyn-Davies & Friedenwald, 1954) and human livers (Dodgson, Spencer & Wynn, unpublished work) are largely unaffected by the aldehyde reagents but are strongly inhibited by phosphate and fluoride. These points support the suggestions of Dodgson & Spencer (1953) and Roy (1953) that two distinct types of arylsulphatases occur in nature.

The functional groups of the enzyme

The arylsulphatase of *Alcaligenes* showed marked variation in the substrate concentration/activity curves at different pH values (see Fig. 4) and a plot of the pK_m values ($-\log_{10} K_m$) against pH gave curves which were very similar for NPS, APS and NCS (Fig. 5). These curves have horizontal portions joined together by positive or negative slopes which are probably 1-unit in character. Dixon (1953) has shown that information about possible functional groups of enzymes can be obtained by consideration of the variation of the dissociation constant (K_s) of the enzyme–substrate

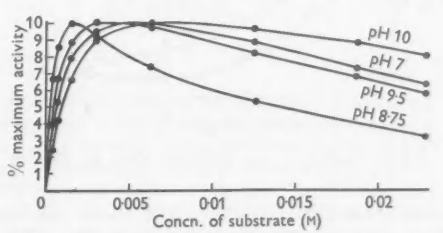


Fig. 4. Effect of pH on the substrate concentration/activity curves for NPS.

complex with pH. Direct determination of K_s was not possible in the present case, but if it can be shown that the K_m values derived for *Alcaligenes* arylsulphatase are a good approximation to the K_s values for this enzyme it is possible to interpret the curves in Fig. 5 according to Dixon's (1953) rules 9 and 10. According to Briggs & Haldane (1925), for the reaction

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products},$$

$K_m = (k_2 + k_3)/k_1$. When k_3 is negligible compared with k_2, K_m is equal to the dissociation constant of the enzyme–substrate complex, $K_m = K_s = k_2/k_1$ (Michaelis & Menten, 1913). For the *Alcaligenes* enzyme both the K_m and the $V_{max.}$ $[k_3(e)]$ values of the enzyme for the three substrates were in the descending order APS, NPS, NCS, and it was thus impossible to assess the magnitude of the contribution of k_3 to $K_m = (k_2 + k_3)/k_1$. However, Dixon (1953) states that in such indeterminate cases rules 9 and 10 can be applied to the pK_m/pH curve in pH regions where the pV/pH curve (Fig. 6) is either (1) horizontal or (2) has non-whole-number slopes or is curved, provided that, in the second case, the pK_m/pH graph has whole-number slopes (e.g. 0- or 1-unit slopes) in the same pH regions.

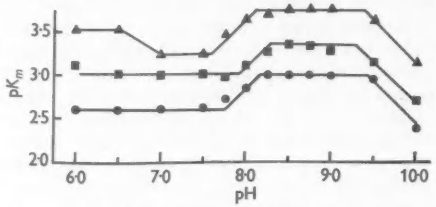


Fig. 5. Variation of pK_m with pH (cf. Dixon, 1953). NCS, ▲; NPS, ■; APS, ●.

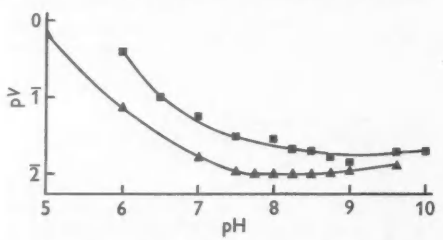


Fig. 6. Variation of pV with pH. NCS, ▲; NPS, ■.

Fig. 6 shows the plot of pV ($pV = -\log_{10} V_{max.}$) against pH for NPS and NCS, from which, using Fig. 5, it may be deduced that Dixon's rules are applicable in the present case.

According to rule 10 the changes in direction shown in the regions of pH 8·2 and 9·4 in Fig. 5 for all three curves reflect pK values of ionizing substrate groupings or substrate-binding enzyme groupings. Fig. 7 shows the titration curves obtained by titrating the free arylsulphuric acids with alkali, and it is apparent that none of these compounds possess groupings with pK values at 8·2 or 9·4. It may thus be presumed that the changes of direction at these two pH values indicate ionizing substrate-binding groups in the enzyme.

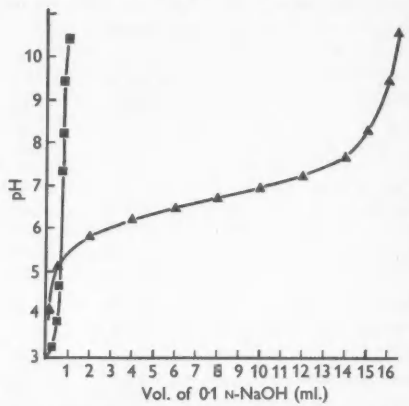


Fig. 7. Titration curves for NCS, ▲; NPS and APS, ■.

The change in direction which occurs in each curve between pH 7·5 and 8 reflects a dissociation of enzyme–substrate complex groupings. Further inflexions in the NCS curve are present at pH values 7·0 and 6·5, the former again due to dissociation of enzyme–substrate complex. The inflexion at pH 6·5 according to rule 10 could be due to dissociation of either enzyme or substrate, but almost certainly the latter, since it is not present in the other two curves. The titration curve for NCS (Fig. 7) shows the presence of a substrate group (presumably the free phenolic group), with a pK in the region of this pH.

A search of the literature gave no clue as to the nature of the substrate-binding enzyme group with pK 8·2, but the group with pK 9·4 could possibly be ϵ- or α-amino, sulphydryl or phenolic, which in proteins dissociate in the region of this pH. Since phosphate did not inhibit enzyme activity (see Table 2) it was assumed that phosphate buffer was not affecting the pK_m/pH curves (cf. Alberty & Massey, 1954).

The action of group-specific protein reagents. It was considered that a study of the effects of certain

group-specific protein reagents might indicate the nature of the ionizing groupings. Equal quantities of the reagent and enzyme were incubated together at 37° for 1 hr., in the presence of 0·1 M phosphate adjusted to the required pH. The mixture was subsequently dialysed overnight, the pH adjusted to 8·75 and the residual enzyme activity determined, using NPS as substrate. Suitable control experiments were made. The results (Table 3) are in contrast with those of Maengwyn-Davies & Friedenwald (1954) for the soluble arylsulphatases of rabbit liver.

amino groups. It must be borne in mind in the interpretation of these results that the presence of at least two substrate-binding groups in the enzyme molecule provides more than one active centre which might be susceptible to the action of protein-specific reagents. Moreover, Table 2 shows that reagents which are known to react with carbonyl groupings (and particularly aldehydic groups) all partially inhibit the *Alcaligenes* enzyme. On similar evidence it has been suggested by Rosenfeld & Ruchelman (1940) that the arylsulphatases of Takadiastase contains an aldehydic

Table 3. *The effect of protein group specific reagents on the arylsulphatase activity of* Alcaligenes metalcaligenes

Residual enzyme activity was measured against NPS under the optimum conditions.

Protein reagent	pH of reaction mixture	Final concn. of reagent	Enzyme activity remaining (%)	Protein groups known to be affected
Glyoxal	7·3	5% (w/v)	6	Guanidyl
Iodoacetic acid	7·4	0·05 M	0	Amino, indole, sulphydryl, phenol
2:4-Dinitrofluorobenzene	7·8	0·0005 M	100	Amino, imidazole, sulphydryl, phenol
p-Chloromercuribenzoic acid	8·0	0·00025 M	51	Sulphydryl
p-Chloromercuribenzoic acid +0·005 M cysteine	8·0	0·0025 M	55	—
p-Chloromercuribenzoic acid +0·005 M glutathione	8·0	0·0025 M	51	—
Diazotized sulphanilic acid	7·1	0·125% (w/v)	0	Amino, imidazole, CH vicinal to OH of tyrosine etc.
Formaldehyde	7·4	20% (w/v)	0	Amino, guanidyl
Formaldehyde + 0·1 g. alanine	11·0	20% (w/v)	0	Amino, guanidyl, indole, amide, sulphydryl, carboxyl
Iodine	8·75	0·005 M	9	Phenolic
Acetic anhydride	5·5 (final pH)	6% (w/v)	7	Amino

DISCUSSION

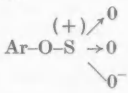
The curves obtained by plotting pK_m against pH have shown the presence in the enzyme of two different substrate-binding groups. The chemical nature of one of these groupings (pK 8·2) is obscure, but the other group (pK at 9·4) might be ϵ- or α-amino, sulphydryl or phenolic, all being groups with similar pK values. The results obtained with the protein-group-specific reagents suggested that sulphydryl might be excluded, since the inhibition (49%) by *p*-chloromercuribenzoic acid could not be reversed by cysteine or glutathione. The experimental results did not permit an unequivocal distinction between phenolic and amino groups, except that the enzyme was completely inhibited by treatment with acetic anhydride under conditions which Fraenkel-Conrat & Fraenkel-Conrat (1950) claim to be specific for substrate-binding grouping. If such a grouping were present in the *Alcaligenes* enzyme it would not necessarily be reflected in the Dixon curves but it might well confuse the experiments with protein group reagents. However, it must be remembered that most of the aldehydic reagents used in the present work would carry some form of charge at the pH values used and their inhibitory effect may simply be due to their combination with an oppositely charged enzyme grouping. These points are being examined further.

Additional information may be derived from the Dixon curves by consideration of rule 9 (Dixon, 1953). Rule 9 states that 'the slope in any pH region is numerically equal to the change of charge on... "desubstration", that is to say it is equal to the number of charges on the left-hand side of the equation

$$E + S \rightleftharpoons ES$$

minus the number of charges on the right-hand side, due regard being paid to algebraic sign'. It has already been pointed out that above pH 7 there is no change in charge on any of the substrates (cf. Fig. 7). Furthermore, the Dixon curves show no evidence of change in charge of enzyme–substrate complex between pH 8 and 10. Since the charges on S and ES are constant between pH 8 and 10 it follows that the enzyme group with pK at 8·2 must be of a type which gains a positive charge (or loses a negative charge) below pH 8·2 to give a +1-unit slope on the pK_m/pH curves. The grouping with pK 9·4 must be one which gains a negative charge (or loses a positive charge) above pH 9·4 to give a −1-unit slope. The possibility that the group with pK at 9·4 might be ϵ- or α-amino is not incompatible with the −1-unit slope obtained above this pH, since the $-NH_2$ group would tend to lose its positive charge above pH 9·4.

It is possible that two oppositely charged groupings in the *Alcaligenes* enzyme are linked with substrate in the form



where the sulphur atom is, to some extent, positively charged, and this possibility is now being investigated further. Wilson & Bergmann (1950), working with cholinesterase, and Gawron, Grelecki & Duggan (1953), working with wheat-germ lipase, suggested that similar linkages existed between these enzymes and their respective substrates.

SUMMARY

1. The arylsulphatase of *Alcaligenes metalcaligenes* has been concentrated and partially separated from nucleic acid and from other enzymes.

2. Some properties of the purified enzyme have been described.

3. The effect of inhibitors and protein-group-specific reagents, together with a study of the variation of the Michaelis constant with pH, has given some indication as to the nature of the substrate-binding groups of the enzymes.

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The Determination of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Animal Tissues

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Sensitive methods have been devised for the separate determination of both oxidized and reduced coenzymes in neutralized acid and alkaline tissue extracts respectively, oxidized coenzymes being relatively stable in dilute acid, whereas reduced coenzymes are relatively stable in dilute alkali (see Schlenk, 1951). Reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), continuously formed in the presence of ethanol and alcohol dehydrogenase or glucose 6-phosphate (G 6-P) and G 6-P dehydrogenase respectively, are coupled with their respective cytochrome *c* reductases and the rate of reduction of cytochrome *c* is followed spectrophotometrically.

$$\left.\begin{array}{l}\mathrm{TPN^+ + glucose\ 6\text{-}phosphate} \xrightarrow[\text{dehydrogenase}]{\text{G 6-P}} \mathrm{6\text{-}phosphogluconate + TPNH + H^+}\\ \mathrm{TPNH + H^+ + 2\ cytochrome\ } c^{3+} \xrightarrow[\text{reductase}]{\text{TPN–cyt. } c} \mathrm{2\ cytochrome\ } c^{2+} + \mathrm{TPN^+ + 2H^+}\end{array}\right\}$$

$$\left.\begin{array}{l}\mathrm{DPN^+ + ethanol} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \mathrm{acetaldehyde + DPNH + H^+}\\ \mathrm{DPNH + H^+ + 2\ cytochrome\ } c^{3+} \xrightarrow[\text{reductase}]{\text{DPN–cyt. } c} \mathrm{2\ cytochrome\ } c^{2+} + \mathrm{DPN^+ + 2H^+}\end{array}\right\}$$

The assay of oxidized and reduced DPN by the present alcohol dehydrogenase–DPN–cytochrome *c* reductase procedure is considerably more sensitive than the estimation of DPN and DPNH with alcohol dehydrogenase and ethanol or acetaldehyde respectively (Racker, 1950). Moreover, our G 6-P dehydrogenase–TPN–cytochrome *c* reductase procedure appears to be the only method available for the estimation of both oxidized and reduced TPN, since no completely reversible TPN-specific dehydrogenase system is at present known.

EXPERIMENTAL

Materials

D-*Glucose 6-phosphate* (*G 6-P*). This was a preparation of the barium salt used previously (Glock & McLean, 1953).

Triphosphopyridine nucleotide (*TPN*). This was made from horse liver by the method of Kornberg & Horecker (1953). It contained 75% TPN (analysed with G 6-P dehydrogenase according to Kornberg, 1950) and no DPN (by the alcohol-dehydrogenase method of Racker, 1950).

Diphosphopyridine nucleotide (*DPN*). This was a commercial preparation (Nutritional Biochemicals Ltd.), subsequently purified by ion-exchange chromatography on Dowex-1-formate according to Kornberg & Pricer (1953). It contained 80% DPN (by the alcohol-dehydrogenase method of Racker, 1950) and no TPN.

Reduced coenzymes. DPNH and TPNH, used in the recovery experiments, were prepared by the enzymic reduction of DPN and TPN with the alcohol-dehydrogenase and glucose 6-phosphate-dehydrogenase systems respectively.

Cytochrome c. This was prepared from horse heart by the method of Keilin & Hartree (1952) and purified by reprecipitation and by ion-exchange chromatography on Amberlite IRC-50 according to Margoliash (1954). The ratio $[D_{550\ m\mu}$ (reduced)$]/[D_{280\ m\mu}$(oxidized)$]$ was 1·165, which corresponds to an Fe content of 0·40%.

Alcohol dehydrogenase. This was prepared from baker's yeast according to Racker (1950).

Glucose 6-phosphate dehydrogenase. The method of Kornberg (1950), using either bottom lager yeast (Anheuser-Busch) or top ale yeast kindly provided by Kongens Bryghus, Copenhagen, was employed.

DPN–cytochrome c reductase. This was prepared from pig hearts by the method of Mahler, Sarkar, Vernon & Alberty (1952), except that the homogenate was centrifuged at 750 *g* for 15 min. (instead of at 2000 *g* for 30 min.) and the top gelatinous mitochondrial layer scraped off and combined with the supernatant. The precipitate from stage R_3 was dissolved in 1% crystalline serum albumin (Armour

and Co. Ltd.) containing 0·01 M glycylglycine, pH 7·6, so as to contain approximately 60 units enzyme (Mahler *et al.* 1952) per ml. We are indebted to T. Walls and Co. Ltd. for a very generous supply of pig hearts.

TPN–cytochrome c reductase. This was prepared from brewer's yeast according to Haas, Horecker & Hogness (1940) up to and including step 4. It was then freeze-dried. Most of this work was carried out with enzymes prepared from Anheuser-Busch bottom ale yeast, but when this was no longer available Löwenbräu (Munich) bottom lager yeast was used. Employing the same definition of units as described by Mahler *et al.* (1952) for DPN–cytochrome *c* reductase, these preparations had an average activity of 10 and 12 units/mg. For the assay of TPN, the enzyme solution used contained approx. 25 units reductase/ml.

Storage of enzymes. All enzyme preparations were stored at $-10°$. For the assay procedure the enzymes, if not already in solution, were dissolved in 1% albumin containing 0·01 M glycylglycine, pH 7·6, and kept at 0°.

Methods

Determination of oxidized and reduced DPN and TPN. Two Potter–Elvehjem glass homogenizers containing respectively 5 ml. 0·1 N-HCl and 5 ml. 0·1 N-NaOH were placed in boiling-water baths. After decapitation of the rat, the tissue under investigation was removed rapidly, blotted free of excess of blood, and two approximately equal portions (50–250 mg.) were weighed on a torsion balance. These were transferred immediately to the two homogenizers, and after 0·5 min. in the hot extracting fluid each was homogenized for 1·5 min. and then rapidly transferred to an ice bath. 0·25 M Glycylglycine, pH 8·7 (1 ml.), was added to each and the volumes were adjusted to 15 ml. by the addition of distilled water. They were then neutralized cautiously with stirring by the dropwise addition of 5 ml. 0·1 N-HCl or 0·1 N-NaOH and finally spun for 30 min. at 0° at 20 000 *g*. The supernatants were poured off, filtered if necessary to remove fat globules, and stored at 0° for the assay of the coenzymes.

Both coenzymes were estimated by following the rate of reduction of cytochrome *c* at 550 mμ. in 1 cm. cells in a Hilger Uvispek spectrophotometer. Oxidized and reduced coenzymes were determined, respectively, in the neutralized acid and alkaline tissue extracts. In the case of the TPN assay, the reaction mixture consisted of 0·5 ml. 0·25 M glycylglycine, pH 8·7, 0·1 ml. 0·05 M G 6-P (Na salt), 0·1 ml. (1 mg.) G 6-P dehydrogenase, 0·1–0·5 ml. neutralized tissue extract and 0·1 ml. TPN–cytochrome *c* reductase in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·001 M cytochrome *c* and readings were taken every minute for 6 min. against a blank cell containing only buffer, water and cytochrome *c*. DPN was determined by a comparable procedure in which G 6-P, G 6-P dehydrogenase and TPN–cytochrome *c* reductase were replaced by 0·1 ml. ethanol, 0·1 ml. (100 μg.) alcohol dehydrogenase and 0·1 ml. DPN–cytochrome *c* reductase.

All results were corrected for the small separately determined non-enzymic reduction of cytochrome *c* by the tissue extracts and by combined reductase, dehydrogenase and substrate. The assay procedures were standardized daily by using four levels of each coenzyme (0·1–0·8 μg. DPN and 0·05–0·4 μg. TPN). The DPN, DPNH, TPN and TPNH contents of the tissue extracts were calculated from the increase in density at 550 mμ. between 1 and 6 min., after correcting for the enzyme and extract blanks.

Test for coenzyme specificity. This was tested for in the DPN–cytochrome *c* reductase system, described above, by replacing DPN with 10 μg. TPNH and in the TPN–cytochrome *c* reductase system by replacing TPN with 10 μg. DPNH.

Tests for interfering enzymes. (*a*) *Enzymic breakdown of DPN and TPN.* Breakdown of DPN by the DPN–cytochrome *c* reductase preparation was determined by incubating the reductase in glycylglycine buffer at room temperature with 0·5 μg. DPN. A control (without DPN) was set up to correct for any loss in reductase activity. After 30 min., 0·5 μg. DPN was added to the control and ethanol, alcohol dehydrogenase and cytochrome *c* to both cells. The rate of reduction of cytochrome *c* was followed for 6 min. as in the assay procedure. A similar experiment was carried out with the TPN–cytochrome *c* reductase preparation and 0·2 μg. TPN.

(*b*) *Enzymic conversion of TPN into DPN.* The usual reaction mixture for the DPN assay system was used, except that DPN was replaced by 1 μg. TPN. Any reduction of cytochrome *c* was followed at 550 mμ.

(*c*) *Pyridine nucleotide transhydrogenase.* This enzyme catalyses the reaction: $TPNH + DPN^+ \rightarrow TPN^+ + DPNH$ (Kaplan, Colowick & Neufeld, 1953), and if present in the TPN–cytochrome *c* reductase preparation would tend to produce low results in the assay of oxidized and reduced TPN in tissue extracts, by converting TPNH into TPN^+. In the test for transhydrogenase in the TPN–cytochrome *c* reductase preparation, the reaction mixture contained only glycylglycine buffer, cytochrome *c*, 2·0 μg. TPNH and 2·0 μg. DPN. The control contained no DPN. The reaction was started by the addition of TPN–cytochrome *c* reductase and the reduction of cytochrome *c* was followed. The transhydrogenase activity of the DPN–cytochrome *c* reductase preparation was tested for in a comparable experiment, although the reversibility of the above transhydrogenase reaction has not so far been demonstrated in animal tissues. The reaction mixture in this case contained only glycylglycine buffer, cytochrome *c*, 5·0 μg. DPNH and 5·0 μg. TPN. The control contained no TPN.

(*d*) *Oxidation of DPNH and TPNH.* This was tested for by following any reoxidation of DPNH (0·1 mg.) or TPNH (0·1 mg.) at 340 mμ. on addition of DPN–cytochrome *c* reductase or TPN–cytochrome *c* reductase respectively.

(*e*) *Cytochrome oxidase.* The cytochrome oxidase activities of the two combined dehydrogenase–cytochrome *c* reductase systems were tested for separately by adding them, in amounts used in the assay procedures, to partially reduced cytochrome *c* and following the reoxidation of reduced cytochrome *c* at 550 mμ.

RESULTS

Extraction procedure

The separate determination of oxidized and reduced coenzymes is possible on account of differences in stability, oxidized coenzymes being relatively stable in dilute acid and reduced coenzymes in dilute alkali (see Schlenk, 1951). This is the basis of methods of estimation of DPN and

DPNH described by Adler & Euler (1936), Euler, Schlenk, Heiwinkel & Högberg (1938), Fisher & Schlenk (1948) and very recently by Holzer, Goldschmidt, Lamprecht & Helmreich (1954). It has also been adopted in the present procedure.

Our results on the stability of oxidized and reduced DPN and TPN in 0·1 M nicotinamide, 0·1 N-HCl and 0·1 N-NaOH at 100 and 85° are shown in Table 1. These results indicate that DPNH and TPNH are unstable both at 85 and 100° in 0·1 M nicotinamide and confirm the extreme lability of oxidized coenzymes in hot alkali and of reduced coenzymes in hot acid. There was somewhat better recovery of DPN and TPN added to hot 0·1 N-HCl at 85° than at 100°, and recoveries of both oxidized and reduced DPN and TPN were good after heating for only 2 min. in 0·1 N-HCl and 0·1 N-NaOH respectively. For these reasons, in the final extraction procedure (see under Methods) the tissue was heated for 0·5 min. at 100° in the extracting medium, followed by homogenization for exactly 1·5 min. The average final temperature of the homogenate was 85–90°. There was no conversion of TPN into DPN on heating for 2 min. in 0·1 N-HCl at 100°.

On applying this 2 min. hot-extraction procedure to the extraction of coenzymes from rat liver (see Table 2), it was found to be immaterial whether the tissue was homogenized at 85° or 100°. The time of heating, however, must not exceed 2 min. In contrast to Holzer *et al.* (1954) it was found immaterial whether the liver homogenates were neutralized before or after centrifuging.

Table 1. *The stability of oxidized and reduced DPN and TPN*

160 μg. DPN+, 80 μg. DPNH, 25 μg. TPN+ or 40 μg. TPNH were added, respectively, to 5 ml. portions of hot extracting medium. After heating for the times stated, tubes were cooled rapidly in ice, and neutralization and estimation of coenzymes carried out as described under Methods.

		Percentage recovery of coenzymes							
		100°				85°			
Extracting medium	Coenzyme	1 min.	2 min.	3 min.	4 min.	1 min.	2 min.	3 min.	4 min.
0·1 M Nicotinamide	DPN+	103	100	95	93	100	100	100	96
	TPN+	91	93	92	80	97	96	—	—
	DPNH	70	72	64	51	69	66	65	60
	TPNH	48	29	10	5	64	45	37	27
0·1 N-HCl	DPN+	100	92	86	82	106	100	102	100
	TPN+	104	95	77	72	104	104	100	100
	DPNH	—	0	—	—	—	—	—	—
	TPNH	—	4	—	—	—	—	—	—
0·1 N-NaOH	DPN+	—	0	—	—	—	—	—	—
	TPN+	—	0	—	—	—	—	—	—
	DPNH	95	92	76	78	92	92	88	79
	TPNH	96	101	86	85	95	96	92	88

Table 2. *Extractions of coenzymes from liver*

Values given for three rat livers expressed as means ±S.E.M. Extraction and assay procedures as described in the Methods section.

		Coenzyme content (μg./g. liver)	
Extracting medium	Coenzyme	100°	85°
0·1 N-HCl	DPN+	370±10	369±13
	TPN+	11± 1	22± 3
0·1 N-NaOH	DPNH	203±11	207±13
	TPNH	215± 7	210± 6

The loss of DPN by adsorption reported by these workers when neutralization preceded spinning can probably be attributed to the relatively large amounts of liver used and to the small final volume of neutralized homogenate.

It has generally been assumed that if tissue extraction is carried out in neutral buffered solutions, it is possible to determine only total DPN and TPN contents. Recently, however, Spirtes & Eichel (1954) have extracted liver with hot phosphate–carbonate buffer, pH 7·4, and have found it possible to determined both DPN and DPNH in the same extract. The values they have reported for rat liver, using alcohol dehydrogenase for the assay of both DPN and DPNH, are considerably higher than those we have found employing DPN–cytochrome *c* reductase. In an attempt to elucidate these discrepancies, we have compared both methods of extraction and methods of estimation. Table 3, which incorporates these results, indicates that the total DPN (DPN^+ + DPNH) contents of phosphate–carbonate extracts of rat liver are consistently higher when determined with alcohol dehydrogenase than with the combined alcohol dehydrogenase–DPN cytochrome *c* reductase procedure described in this paper. This

Table 3. *Comparison of methods of extraction and estimation of coenzymes*

Extracting medium	Method of estimation	Coenzyme content* (μg./g. liver) DPN+	DPNH	DPN+ + DPNH	TPN+ + TPNH	DPN+/DPNH
Phosphate–carbonate†	*A*	386±14	339±18	725±27	—	1·1±0·05
	B	—	—	522±37	203±11	—
0·1 N-HCl or 0·1 N-NaOH	*B*	364±13	200±11	564±18	207± 9	1·8±0·1

* Values for four rat livers expressed as means±S.E.M.

† Method of Spirtes & Eichel (1954).

A, Spectrophotometric estimation with alcohol dehydrogenase; *B*, spectrophotometric estimation at 550 mμ. as described in the Methods section.

is due chiefly to the much higher DPNH values obtained with the former method. On the other hand, our figures for both total DPN and DPN^+/DPNH quotients of rat livers are in fairly good agreement with those of Holzer *et al.* (1954), who employed similar acid and alkaline extraction procedures but estimated DPN and DPNH with alcohol dehydrogenase. It is difficult to account for the high DPNH levels obtained by the procedure of Spirtes & Eichel (1954). They might be attributable to the lack of absolute coenzyme specificity of alcohol dehydrogenase (Singer & Kearney, 1954*a*), which would imply the presence of more biologically active pyridine derivatives in phosphate extracts of liver than in acid or alkaline extracts.

Coenzyme specificity

The coenzyme specificity of both DPN– and TPN–cytochrome *c* reductase systems employed in the assay procedure was found to be absolute (see under Methods).

pH/*activity curves*

The pH/activity curves for the DPN and TPN assay systems in 0·05 M glycylglycine are given in Fig. 1. The TPN system has a broad optimum between approximately pH 8·3 and 9·9 and the DPN system from pH 7·6 to 9·0. The final assay procedure for both coenzymes was carried out in 0·05 M glycylglycine at pH 8·7. Although this is not the optimum pH for either G 6-P dehydrogenase or alcohol dehydrogenase, a large excess of these enzymes was employed so that the rate of reduction of neither TPN nor DPN was limiting.

The effect of various buffers and salts on the activity of the two reductase systems is shown in Table 4. Vernon, Mahler & Sarkar (1952) have shown that purified DPN–cytochrome *c* reductase is inhibited considerably by various ions, including phosphate, chloride, sulphate, Mg^{++} and Na^+.

These results indicate the advisability of employing glycylglycine as buffer in the present assay procedure and of omitting $MgCl_2$ from the TPN method. Lack of inhibition of the DPN system by NaCl and sulphate can probably be attributed to the relative impurity of the reductase preparations.

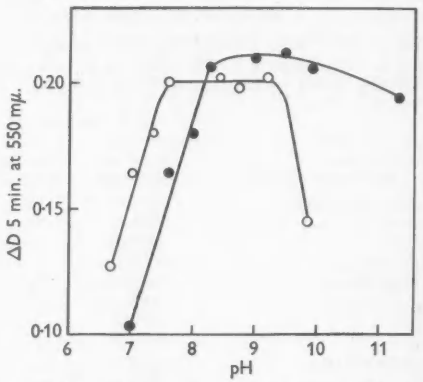

Fig. 1. pH/activity curves of the DPN and TPN assay systems in glycylglycine buffer. Activities determined as described in the Methods section. ○, 6 units of DPN–cytochrome *c* reductase, 0·3 μg. DPN; ●, 2 units of TPN–cytochrome *c* reductase, 0·3 μg. TPN.

Table 4. *Effect of certain buffers and salts on the activity of the DPN and TPN assay systems*

Results expressed as percentage of activity of assay system measured in 0·05 M glycylglycine, pH 8·7, as described in the Methods section. Activities in the presence of added salts determined in 0·05 M glycylglycine, pH 8·7.

Final concn.	Percentage activity: DPN assay system	TPN assay system
0·05 M Glycylglycine buffer, pH 8·7	100	100
0·02 M Diol buffer, pH 8·7*	95	94
0·01 M Phosphate buffer, pH 8·3†	—	30
0·02 M-NaCl‡	99	102
0·02 M-$MgCl_2$§	—	37
0·10 M-$(NH_4)_2SO_4$‖	100	—

* 2-Amino-2-methyl-1:3-propanediol buffer ('Diol' buffer) as used by Vernon *et al.* (1952).

† Assay conditions of Haas *et al.* (1942).

‡ The final concn. if 1 ml. neutralized tissue extract used in assay.

§ The optimum concn. for G 6-P dehydrogenase activity.

‖ The final concn. in DPN assay due to addition of alcohol dehydrogenase.

Variations in cytochrome c concentration

The effect of varying the concentration of cytochrome *c* on the assay of DPN and TPN by the two dehydrogenase–reductase systems is shown in Fig. 2. It is obvious that the concentration of cytochrome *c* employed in the assay procedure (0·1 ml. 0·001 M cytochrome *c* in a total volume of 2·5 ml.) is optimum for both systems with the particular coenzyme concentrations used.

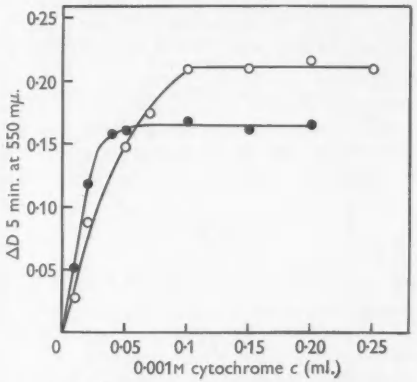

Fig. 2. Effect of variations in cytochrome *c* concentration on the activities of the DPN and TPN assay systems. Activities determined as described in the Methods section. ○, 6 units of DPN–cytochrome *c* reductase, 0·3 μg. DPN; ●, 2·4 units of TPN–cytochrome *c* reductase, 0·2 μg. TPN.

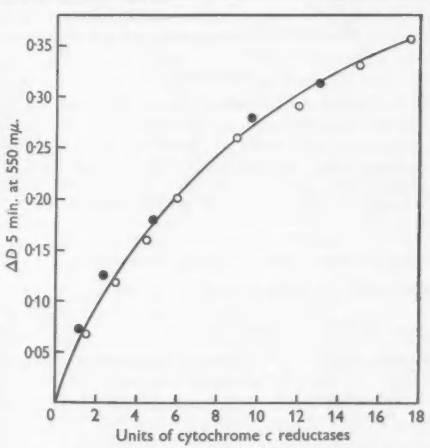

Fig. 3. Effect of variations in the concentration of DPN–cytochrome *c* reductase and TPN–cytochrome *c* reductase. Activities determined as described in the Methods section. ○, DPN assay system with 0·35 μg. DPN; ●, TPN assay system with 0·14 μg. TPN.

Variations in cytochrome c reductase concentrations

The effect of variations in concentration of DPN–cytochrome *c* reductase and TPN–cytochrome *c* reductase on the assay of DPN and TPN respectively is shown in Fig. 3. It is apparent that

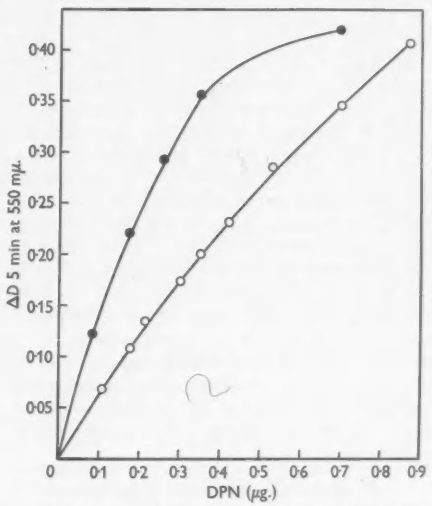

Fig. 4. DPN concentration curves. ○, 6 units of DPN–cytochrome *c* reductase; ●, 18 units of DPN–cytochrome *c* reductase.

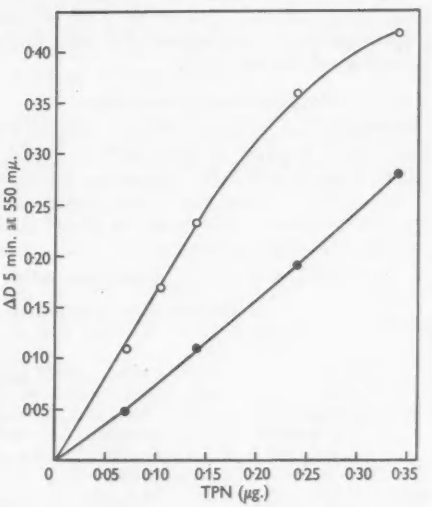

Fig. 5. TPN concentration curves. ●, 2·4 units of TPN–cytochrome *c* reductase; ○, 7·2 units of TPN–cytochrome *c* reductase.

the sensitivity of both methods of assay can be increased by employing more reductase.

Variations in coenzyme concentrations

The effects of varying the concentrations of DPN and TPN on the rate of reduction of cytochrome *c* by the two assay systems are shown in Figs. 4 and 5 respectively. Two levels of reductase were used, the lower levels (6 units of DPN–cytochrome *c* reductase and 2·4 units of TPN–cytochrome *c* reductase) corresponding to the approximate concentrations of reductases employed in the assay procedures. Although these lower levels of reductase are not optimum, the rate of reduction of cytochrome *c* is, nevertheless, directly proportional to coenzyme concentration up to a ΔD at 550 mμ. of about 0·3, corresponding to approximately 30 % reduction of the cytochrome *c*. The advantage of using these suboptimum concentrations of reductases in the assay procedures are, first, that the range of concentration of coenzyme that can be estimated is extended and, secondly, that the non-enzymic reduction of cytochrome *c* by the reductase preparation itself is minimized. The range of the methods can also be extended by increasing the concentration of cytochrome *c*.

Variations in volume of tissue extract

The rate of reduction of cytochrome *c* was found to be proportional to the volume of neutralized tissue extract used in both DPN and TPN assay procedures provided that not more than approximately 30 % of the cytochrome *c* is reduced. Typical results for neutralized acid and alkaline extracts are shown in Fig. 6.

Recovery of added coenzymes

The results incorporated in Table 5 indicate that there is good recovery of DPN, DPNH, TPN and TPNH when added both during the extraction procedure and to the final assay system. The quantitative recovery of coenzymes added to the assay systems indicates that neutralized liver extracts contain no substances that alter the rate of reduction of cytochrome *c* by the reductase systems. This also applies to the other tissue extracts investigated.

Lack of interference by other enzymes

The present assay procedures would be completely invalidated if the systems were contaminated with any of a variety of interfering enzymes. These include enzymes capable of breaking down DPN or TPN, of converting TPN into DPN, of oxidizing DPNH and TPNH, transhydrogenase

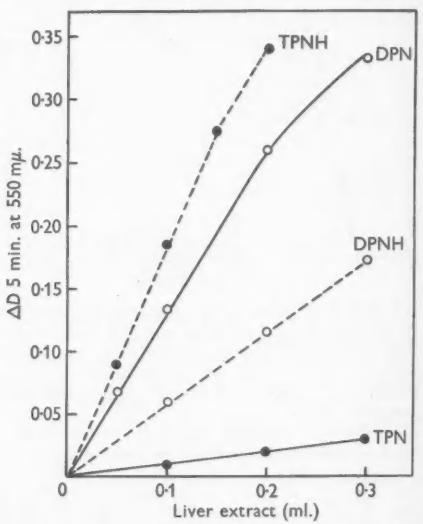

Fig. 6. Direct proportionality between the volume of liver extract and the rate of reduction of cytochrome *c*. Extraction and assay as described in the Methods section, using approximately 200 mg. rat liver. —, Neutralized acid extract; – – –, neutralized alkaline extract; ○, DPN or DPNH; ●, TPN or TPNH.

Table 5. *Recovery of coenzymes added to liver during extraction and assay procedures*

Extraction and assay procedures as described in the Methods section.

		Recovery of coenzymes*			
		Added during extraction†		Added to assay system	
Extracting medium	Coenzyme	μg. added	Percentage recovery	μg. added	Percentage recovery
0·1 N-HCl	DPN+	60	101±6	0·4	100±1
	TPN+	40	98±7	0·1	100±2
0·1 N-NaOH	DPNH	60	95±8	0·4	100±2
	TPNH	40	93±5	0·1	99±3

* Values for three rat livers expressed as means±S.E.M.

† Coenzymes added to hot extracting medium immediately after introduction of approximately 200 mg. liver.

and cytochrome oxidase. For this reason the dehydrogenase–cytochrome *c* reductase systems used in the assays were carefully tested for the presence of these enzymes, as described under Methods. They were all found to be absent from both systems. Since diaphorase, which reacts sluggishly with oxygen (see Singer & Kearney, 1954*b*), would be included in the group of enzymes capable of re-oxidizing DPNH and TPNH, and these were found to be absent or inactive under our conditions of assay, it is obvious that the diaphorase activity of the DPN–cytochrome *c* reductase does not interfere with the assay procedure. In addition, Singer & Kearney (1950) have reported that various non-specific flavins can catalyse the non-enzymic reduction of cytochrome *c* by DPNH. These cannot be present in our tissue extracts, since the rate of reduction of cytochrome *c* by the extracts alone was not increased on the addition of DPNH.

DISCUSSION

Quantitative methods have been described for the determination of both oxidized and reduced DPN and TPN in animal tissues. These involve coupling reduced coenzymes with their respective cytochrome *c* reductases and following spectrophotometrically the rate of reduction of cytochrome *c*. These methods are so sensitive that it has been possible to determine both oxidized and reduced DPN and TPN even in tissues with very low coenzyme contents, without preliminary concentration of the tissue extracts. As far as we are aware tissue-coenzyme levels have not previously been estimated in this way, although TPN–cytochrome *c* reductase has been used for estimating TPN in pure solution (Haas, Harrer & Hogness, 1942). Apart from the recently published figures of Holzer *et al.* (1954), Frunder (1954) and Spirtes & Eichel (1954) for the DPN and DPNH contents of liver (determined spectrophotometrically with alcohol dehydrogenase), very few reliable figures are available for both DPN and DPNH contents of animal tissues (see Schlenk, 1951), and even fewer for TPN. There are none for TPNH. This scarcity of results can be attributed chiefly to errors in the extraction procedure, to the use of unreliable coenzyme standards and to the relatively low TPN content of most tissues. Moreover, the manometric methods available are not completely coenzyme specific. Thus Adler, Elliott & Elliott (1940) showed that conversion of TPN into DPN occurs in the apozymase fermentation system, and Negelein & Wulff (1937) demonstrated that 'old yellow enzyme', used as oxygen carrier in the *Zwischenferment* system of Warburg, Christian & Griese (1935), reacts with both DPNH and TPNH.

Singer & Kearney (1954*a*) have shown that Co III can replace DPN in the alcohol dehydrogenase and lactic dehydrogenase systems and reduced Co III can replace DPNH in the DPN–cytochrome *c* reductase system. This presumably means that published values for DPN contents of animal tissues involving the use of any of these enzyme systems are too high, owing to inclusion of Co III. This coenzyme is widely distributed in animal tissues, the concentration being highest in kidney and liver (Singer & Kearney, 1953).

Our most interesting finding is that whereas in liver DPN is present mainly in the oxidized form the reverse is true of TPN, which is present almost exclusively in the reduced form (see Table 2 and Fig. 6). Thus the average $DPN^+/DPNH$ quotient in liver is 1·8, whereas the $TPN^+/TPNH$ quotient is as low as 0·03. This is difficult to interpret and cannot be explained by the activity of the pyridine nucleotide transhydrogenase system, which favours the formation of DPNH (Kaplan *et al.* 1953).

SUMMARY

1. Sensitive methods have been described for the separate determination of DPN^+, DPNH, TPN^+ and TPNH in animal tissues. These involve coupling reduced coenzymes with their respective cytochrome *c* reductases and following the rate of reduction of cytochrome *c* spectrophotometrically.

2. By these methods it is possible to estimate accurately as little as 0·1 μg. DPN or TPN.

3. DPN is present in rat liver mainly in the oxidized form, whereas TPN is present mainly in the reduced form. Average DPN^+, DPNH, TPN^+ and TPNH values are, respectively, 370, 204, 6 and 205 μg. coenzyme/g. liver.

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Levels of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Animal Tissues

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The preceding paper (Glock & McLean, 1955) describes sensitive methods for the separate determination of oxidized and reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in animal tissues. Reduced coenzymes are coupled with their respective cytochrome *c* reductases and the rate of reduction of cytochrome *c* is followed spectrophotometrically. These methods have now been applied to the estimation of DPN^+, DPNH, TPN^+ and TPNH in a variety of animal tissues.

EXPERIMENTAL

The preparation of substrates and enzymes and the estimation of coenzymes were carried out as described previously (Glock & McLean, 1955). Oxidized and reduced coenzymes were determined, respectively, in neutralized acid and alkaline tissue extracts.

RESULTS

Our results for the levels of oxidized and reduced DPN and TPN in a variety of animal tissues are incorporated in Table 1. The rate of reduction of cytochrome *c* was in all cases directly proportional to the volume of tissue extract. The presence of relatively high concentrations of ascorbic acid in the neutralized acid extracts of adrenals does not invalidate the assay procedures, since, although ascorbic acid reduces cytochrome *c* directly, the rate of reduction is so rapid that it is virtually complete before the first spectrophotometric reading is taken.

In all the tissues investigated, the total DPN concentrations ($DPN^+ + DPNH$) are considerably higher than the total TPN concentrations ($TPN^+ + TPNH$). Moreover, whereas DPN is present chiefly in the oxidized form, TPN on the contrary is present mainly in the reduced form. Thus the $DPN^+/DPNH$ quotients are in all cases greater than 1 and the $TPN^+/TPNH$ quotients always considerably less than 1. In many tissues the levels of oxidized TPN are negligible. The highest total concentration of TPN is found in liver but fairly high concentrations also occur in adrenals, lactating mammary glands, ovary and kidney. Most of the other tissues examined contain very little TPN either in the oxidized or reduced form. The low values in brain and voluntary muscle (diaphragm), amounting to less than 5% of the total DPN concentration, are particularly interesting. The most variable results were obtained with the adrenals both for the total concentrations of DPN and TPN and for the proportions of oxidized to reduced coenzymes. This may prove to be related to the physiological state of activity of the gland, particularly since both DPN and TPN have been shown to be necessary for various steroid interconversions in adrenal homogenates (Hayano, 1954; Hayano & Dorfman, 1953; Plager & Samuels, 1953). The high TPN levels in ovary might also be associated with steroid metabolism.

Table 1. *Levels of oxidized and reduced coenzymes in animal tissues*

Figures in parentheses represent number of animals.

Tissues	Coenzyme content (μg./g. tissue)					
	DPN+	DPNH	DPN+ + DPNH	TPN+	TPNH	TPN+ + TPNH
Rat liver (6)	370±13	204±9	574±17	6±1	205±6	211±6
Rat adrenal (5)	315±136	154±45	469±134	17±9	116±24	133±24
Rabbit adrenal (1)	295	117	412	<2	62	62
Rabbit adrenal cortex (1)	356	133	489	14	68	82
Rat diaphragm (6)	289±7	138±9	427±14	<2	13±1	13±1
Rat cardiac muscle (3)	299±15	184±38	483±23	4±1	33±5	36±2
Rat kidney (3)	223±12	212±54	435±60	3±1	54±1	57±2
Rat mammary gland (18 days' lactation) (3)	227±9	83±3	310±10	<2	51±13	51±13
Guinea-pig ovary (1)	214	38	252	6	116	122
Rabbit ovary (1)	181	34	215	<2	42	42
Rat brain (3)	133±6	88±36	221±42	<2	8±3	8±3
Guinea-pig brain cortex (1)	155	67	222	8	16	24
Rat spleen (3)	135±12	61±15	196±18	<2	12±3	12±3
Rat thymus (3)	116±17	35±13	151±20	<2	12±2	12±2
Rat lung (2)	108	52	160	9	18	27
Rat pancreas (2)	115	78	193	<2	12	12
Guinea-pig thyroid (1)	126	30	156	<2	<2	<2
Rat seminal vesicles (1)	128	11	139	<2	12	12
Rat ventral prostate (2)	80	17	97	<2	11	11
Rat testis (1)	80	71	151	<2	6	6
Rat placenta (20-day pregnancy) (2)	90	11	101	<2	3	3
Rat blood* (3)	55±3	36±10	91±8	5±2	3±2	8±3
Rabbit blood* (2)	33	4	37	3	<2	3

* Coenzymes expressed in μg./ml. whole blood.

Our figures for the total DPN content of lactating mammary glands are in good agreement with those obtained by Ringler, Becker & Nelson (1954) in the guinea pig, using alcohol dehydrogenase, and our combined values for the DPN+ + DPNH + TPN+ + TPNH contents of muscle, heart, kidney, spleen, lung and pancreas agree moderately well with the figures for total pyridine nucleotide contents of these tissues determined polarographically by Carruthers & Suntzeff (1953).

DISCUSSION

The values reported in this paper for the DPN+, DPNH, TPN+ and TPNH contents of animal tissues are, with a few exceptions, the first to be published. Apart from recent figures for the DPN+ and DPNH contents of liver, which are discussed in the previous paper (Glock & McLean, 1955), very few reliable figures are available (see Schlenk, 1951) and there are none for TPNH.

It is of interest that whereas in all the tissues studied DPN is present mainly in the oxidized form, the reverse is true of TPN, which is present predominantly and sometimes exclusively in the reduced form. The steady-state ratios of oxidized to reduced coenzymes are presumably controlled both by concentrations of substrates and of competing dehydrogenases. Chance (1954) has found large variations in the steady-state level of DPN+/DPNH in respiring yeast cells, and has concluded that this ratio is governed chiefly by competing dehydrogenases, in this case chiefly by triosephosphate dehydrogenase and α-glycerophosphate dehydrogenase. The steady-state quotient of DPN+/DPNH is also presumably controlled by oxidative phosphorylation. Thus Lehninger (1951) and Vishniac & Ochoa (1952) have shown that oxidative phosphorylation of ADP to ATP can be coupled with the oxidation of DPNH. This would explain the oxidation of reduced pyridine nucleotides recently observed by Connelly & Chance (1954) in the contracting sartorius muscle of the frog, and the oxidation of reduced pyridine nucleotides on addition of low concentrations of ADP to rat-liver mitochondrial preparations respiring in the presence of β-hydroxybutyrate (Chance & Williams, 1954). It seems probable, although not yet proved, that oxidative phosphorylation can also be coupled with the oxidation of TPNH. The very low TPN+/TPNH quotients in all the tissues examined presumably indicates that the TPN-dependent dehydrogenase systems are relatively irreversible and that the steady-state levels of TPN+/TPNH are controlled more by these dehydrogenases than by TPN–cytochrome *c* reductase and glutathione reductase, which cause reoxidation of TPNH. It is of interest in this connexion that Hogeboom & Schneider (1950) found the TPN–cytochrome *c* reductase activity of mouse

liver to be considerably less than previously reported values for DPN–cytochrome c reductase.

The total TPN contents of the tissues investigated show some rough positive correlation with levels of activity of glucose 6-phosphate and 6-phosphogluconate dehydrogenases (see Glock & McLean, 1954) and with glutathione reductase (see Rall & Lehninger, 1952).

SUMMARY

1. The DPN^+, DPNH, TPN^+ and TPNH contents of a variety of animal tissues have been determined.

2. In all the tissues investigated DPN is present mainly in the oxidized form, whereas TPN is present chiefly and sometimes exclusively in the reduced form.

3. High total concentrations of both DPN and TPN are found in liver, adrenals, kidney and lactating mammary glands. The high level of TPN in ovary and the low levels in brain and voluntary muscle are also of interest.

We are indebted to Professor F. Dickens, F.R.S., for interest, and to Miss Audrey Bowles for valuable technical assistance. This work was carried out during the tenure of the Hugh Percy Noble Scholarship by G.E.G. and has been in part financed by a grant to the Medical School from the British Empire Cancer Campaign.

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A Preliminary Investigation of the Hormonal Control of the Hexose Monophosphate Oxidative Pathway

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An earlier investigation (Glock & McLean, 1954) of levels of activity of enzymes of the hexose monophosphate oxidative pathway in mammalian tissues and tumours suggested that this pathway is probably under hormonal control. In addition, recent preliminary experiments (Glock & McLean, 1955) have shown that the levels of activity of glucose 6-phosphate and 6-phosphogluconate dehydrogenases are significantly reduced in the livers of rats in alloxan diabetes. These experiments on diabetes have now been extended and the effects of starvation, variations in food intake and administration of growth hormone, thyroxine and thiouracil have also been investigated. A later paper will deal with the effects of sex hormones, hypophysectomy and adrenalectomy.

EXPERIMENTAL

Materials

D-Glucose 6-phosphate (G 6-P) and 6-phosphogluconate (6-PG). These were preparations of the barium salts used previously (Glock & McLean, 1953).

Triphosphopyridine nucleotide (TPN). This was made from horse liver by the method of Kornberg & Horecker (1953). It contained 75% TPN (analysed with G 6-P dehydrogenase according to Kornberg, 1950) and no diphosphopyridine nucleotide (DPN) (by the alcohol-dehydrogenase method of Racker, 1950).

Methods

Estimation of G 6-P and 6-PG dehydrogenase activities. Liver and voluntary-muscle dehydrogenase activities were determined spectrophotometrically by following the rate of reduction of TPN at 340 mμ. in 1 cm. cells in a Hilger

Uvispek spectrophotometer as described previously (Glock & McLean, 1953). In the assay of G 6-P dehydrogenase activity, the reaction mixture consisted of 0·5 ml. 0·25 M glycylglycine, pH 7·6, 0·5 ml. 0·1 M-$MgCl_2$, 0·1 ml. 6-PG dehydrogenase [dialysed $(NH_4)_2SO_4$ liver fraction], 0·1 ml. liver supernatant or 0·5 ml. muscle supernatant and 0·2 mg. TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·05 M G 6-P to both cells, the blank being devoid of TPN. In the assay of 6-PG dehydrogenase activity, the $(NH_4)_2SO_4$ liver fraction was omitted, G 6-P was replaced by 0·1 ml. 0·05 M 6-PG and the reaction was carried out in glycylglycine buffer at both pH 7·6 and pH 9·0. A unit of enzyme activity is defined as the quantity of enzyme which reduces 0·01 μ-mole TPN/min. at 20°.

Determination of nucleic acids. In some of the experiments both the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contents of the livers were determined. The extraction procedure adopted was that of Schneider (1946). DNA was estimated by a modification of the diphenylamine method of Dische (1930) as described by Racker (1952), except that the readings were taken at 600 mμ. RNA was determined by the orcinol method of Mejbaum (1939), the time of heating being increased to 45 min. The standard RNA was a commercial yeast preparation purified according to Fletcher, Gulland, Jordan & Dibben (1944). The standard DNA was a preparation of the sodium salt prepared by the late Professor J. Gulland and his colleagues and was kindly given by Dr J. M. Creeth. According to the notation suggested by Chargaff & Zamenhoff (1948), the atomic extinction coefficients, ϵ(P), of samples treated with hot trichloroacetic acid as in the extraction procedure were 10 910 for RNA and 8350 for DNA. These values are in fairly good agreement with those quoted by Ogur & Rosen (1950) and Robinson (1952).

Nitrogen contents of tissues. These were determined by a micro-Kjeldahl procedure.

Dietary procedure. In general, albino rats between 160 and 180 g. in weight were used and, with the exception of the growth hormone experiments, were all males. In order to accustom the animals to the diet (that of Greenbaum & Young, 1953), they were fed this for 7 days before the experiment was started. In all the experiments, the control and treated group of animals were pair-fed.

Effect of variations in food intake and of starvation. In this experiment, the control group was fed *ad lib.* (20–21 g./rat/day), three groups received respectively 5, 10 and 15 g. of food daily for 8 days, and one group was starved for 48 hr. before being killed.

Treatment with growth hormone. Two samples of crystalline growth hormone were used, both being prepared by the method of Wilhelmi, Fishman & Russell (1948). One (GH 1) was prepared and kindly provided by Dr A. L. Greenbaum and the other (GH 2) was a commercial preparation presented by Dr Steelman of Armour and Co. Ltd. Adult female rats were subjected to both short-term and long-term treatment with growth hormone. In the short-term experiments, 2 mg. GH 1 were injected intraperitoneally into albino rats and the animals killed after 5 hr. In the long-term experiments, 400 μg. GH 1 or 500 μg. GH 2 were injected daily subcutaneously into hooded Norway or albino rats for 8 or 10 days respectively before being killed.

Treatment with thyroxine. These animals received a daily subcutaneous injection of 0·5 mg. DL-thyroxine (as the Na salt) for 8 days.

Treatment with thiouracil. This group of animals was fed on the diet supplemented with 0·1% thiouracil for 3 weeks.

Production of alloxan diabetes. After preliminary starvation for 48 hr. the rats were made diabetic by the subcutaneous injection of alloxan according to Kass & Waisbren (1945) and killed 10 days after the injection. The urinary excretion of reducing sugar was determined daily.

In vitro effects of hormones. The *in vitro* effects of growth hormone (GH 1), crystalline insulin (Boots Pure Drug Co. Ltd.) and DL-thyroxine (Roche) were tested on the G 6-P and 6-PG dehydrogenase activities of dialysed liver supernatants. Dehydrogenase activities were determined as described above, except that the reactions were allowed to proceed at room temp. for 15 min. and the $(NH_4)_2SO_4$ liver fraction containing 6-PG dehydrogenase was omitted from the G 6-P dehydrogenase assay system. The final concentrations of hormones employed were 0·001 M thyroxine, 200 μg. growth hormone/ml. or 0·4 or 1·0 unit of insulin/ml.

RESULTS

Starvation and variations in food intake

The results for the ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) contents and for the G 6-P and 6-PG dehydrogenase activities of liver are shown in Table 1. The DNAP/g. liver increased progressively with decreasing food intake and was markedly increased after 48 hr. starvation, but the nitrogen and RNAP contents/g. liver were relatively constant. On the other hand, the total DNAP contents in mg./liver were relatively constant in the different groups, whereas the total RNAP in mg./liver showed a steady decline with decreasing food intake (see Fig. 1). Similar effects on the total RNAP and DNAP contents of rat liver after 72 hr. starvation have been reported by Thomson, Heagy, Hutchinson & Davidson (1953). The G 6-P and 6-PG dehydrogenase activities expressed in units/g. liver were scarcely affected by restricted food intake, whereas the total units of both dehydrogenases in the whole liver showed a steady fall (see Fig. 1). A similar pattern of change of G 6-P and 6-PG dehydrogenase activities is obtained if the dehydrogenase activities are expressed in units/mg. DNAP. It is of interest that the rate of decrease of total liver RNAP parallels the decline in total dehydrogenase activities. After 48 hr. starvation, the total dehydrogenase activities were close to those of the group fed 5 g. food/day, namely, approximately 50% of the control values.

Alloxan diabetes

The RNAP and DNAP contents and the levels of activity of G 6-P and 6-PG dehydrogenases in the livers of alloxan-diabetic and pair-fed control rats are shown in Table 2. There is a very significant decrease in the levels of activity of both dehydrogenases in the livers of the diabetic rats which is accentuated if the results are expressed as total

Table 1. *Effect of starvation and variations in food intake on levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat liver*

Results expressed as means ± S.E.M. For details, see Methods section.

Daily food intake	Unlimited (Average 20·5 g.)	15 g.	10 g.	5 g.	Starved for 48 hr.
No. of animals	12	6	6	6	6
Initial body wt. (g.)	164±1	163±2	162±1	161±2	155±3
Final body wt. (g.)	187±3	166±3	147±4	119±3	141±3
Liver wt. (g.)	7·99±0·25	6·86±0·25	5·46±0·20	4·30±0·14	5·39±0·14
Liver nitrogen (mg./g.)	35·2±0·3	36·0±0·5	38·2±0·2	40·8±0·3	38·9±0·4
DNAP (μg./g. liver)	259±10	269±14	313±13	383±12	321±16
RNAP (μg./g. liver)	606±13	542±30	574±12	543±21	632±11
Units of enzyme/g. liver					
G 6-P dehydrogenase, pH 7·6	61±5	54±2	52±4	50±3	39±3
6-PG dehydrogenase, pH 9·0	163±7	137±7	158±19	159±9	148±8
6-PG dehydrogenase, pH 7·6	80±3	60±3	57±7	57±3	76±4
Units of enzyme/mg. DNAP:					
G 6-P dehydrogenase, pH 7·6	238±19	200±11	166±9	133±9	120±8
6-PG dehydrogenase, pH 9·0	637±37	515±35	510±70	415±14	462±17
6-PG dehydrogenase, pH 7·6	307±18	222±16	182±25	148±5	238±9

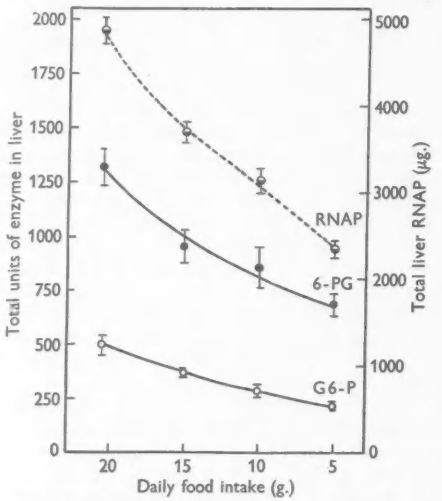

Fig. 1. Effect of variations in food intake on the levels of activity of G 6-P and 6-PG dehydrogenases and on the ribonucleic acid content of rat liver. ○, G 6-P dehydrogenase, pH 7·6; ●, 6-PG dehydrogenase, pH 9·0; ◒, RNAP. The vertical lines represent twice the S.E.M.

units of enzyme in the whole liver. The total RNAP (μg./whole liver) is also significantly lower in the diabetic animals. This latter finding is not in agreement with Thomson *et al.* (1953), who found no significant alterations in either total DNAP or RNAP contents of liver in alloxan diabetes. The dehydrogenase activities have not been expressed on a DNA basis, since Diermeier & Di Stefano (1954) have shown that the DNA content/liver cell nucleus is increased in alloxan-diabetic rats.

Similar changes in dehydrogenase levels to those shown in Table 2 were obtained in a preliminary experiment in which the animals were less diabetic, excreting on an average only 2 g. reducing sugar/day.

Treatment with growth hormone

The effects of short- and long-term treatments with growth hormone on the levels of activity of G 6-P and 6-PG dehydrogenases of rat liver are shown in Tables 3 and 4. Short-term treatment, in which the animals were killed 5 hr. after the injection of 2 mg. of growth hormone, produced no significant changes in the levels of either dehydrogenase (see Table 3). After long-term treatment with two different crystalline growth-hormone preparations (see Table 4), 6-PG dehydrogenase activities expressed in units/g. liver were significantly reduced in both experimental groups. There was also a marked increase in the average G 6-P dehydrogenase activity in the first group. When expressed as total units/liver or on a DNA basis, however, none of these differences was statistically significant. This can be attributed to wide variations in the individual dehydrogenase activities, which are encountered only in groups of female rats.

Thyroxine treatment

The levels of activity of G 6-P and 6-PG dehydrogenases in the liver and voluntary muscles of control and pair-fed thyroxine-treated rats are given in Table 5. There is a very significant increase in the levels of activity of both dehydrogenases in the livers of the thyroxine-treated animals. Although it was previously shown (Glock & McLean, 1954) that muscle G 6-P and 6-PG dehydrogenase levels are very low, they were, nevertheless, determined in this experiment on account of the peripheral action of thyroxine. No significant differences were found.

Table 2. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in the livers of control and diabetic rats*

Results expressed as ±S.E.M. For details, see Methods section.

	Control group	Alloxan-treated group	*P*
No. of animals	6	6	—
Initial body wt. (g)	170±2	170±1	—
Final body wt. (g.)	202±4	146±8	—
Liver wt. (g.)	8·6±0·5	6·6±0·5	—
Average daily sugar excretion (g.)	—	6±1	—
Liver nitrogen (mg./g.)	34·6±0·9	38·3±0·8	0·013
DNAP (μg./whole liver)	1738±73	1651±103	0·502
RNAP (μg./whole liver)	4628±245	3561±216	0·010
Units of enzyme/g. liver:			
G 6-P dehydrogenase, pH 7·6	149±9	58±6	<0·001
6-PG dehydrogenase, pH 9·0	187±13	82±4	<0·001
6-PG dehydrogenase, pH 7·6	111±4	40±4	<0·001
Total units of enzyme in liver:			
G 6-P dehydrogenase, pH 7·6	1296±144	377±41	<0·001
6-PG dehydrogenase, pH 9·0	1612±175	544±68	<0·001
6-PG dehydrogenase, pH 7·6	952±69	261±25	<0·001

Table 3. *Effect of short-term treatment with growth hormone on the levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat liver*

Female albino rats injected intraperitoneally with 2 mg. growth-hormone preparation 1 (see Methods section) and killed 5 hr. later. Results expressed as means±S.E.M.

	Control group	Growth-hormone-treated group	*P*
No. of animals	5	5	—
Body wt. (g.)	160±1	161±2	—
Liver wt. (g.)	6·11±0·16	6·39±0·21	—
Units of enzyme/g. liver:			
G 6-P dehydrogenase, pH 7·6	106±18	82±6	0·230
6-PG dehydrogenase, pH 9·0	309±32	338±23	0·480
Total units of enzyme in liver:			
G 6-P dehydrogenase, pH 7·6	656±129	525±34	0·345
6-PG dehydrogenase, pH 9·0	1914±227	2147±119	0·351

Table 4. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in the livers of control and growth-hormone-treated rats*

For description of growth-hormone preparations see under experimental section. Results expressed as means±S.E.M.

	Control group	Growth-hormone-treated group 1*	*P*	Control group	Growth-hormone-treated group 2†	*P*
No. of animals	5	5	—	11	11	—
Initial body wt. (g.)	158±4	157±4	—	182±6	184±5	—
Final body wt. (g.)	164±7	180±4	—	192±5	205±4	—
Liver wt. (g.)	5·95±0·51	7·50±0·81	—	6·53±0·27	7·16±0·26	—
Total DNAP (μg./liver)	1104±142	1403±124	0·148	—	—	—
Total RNAP (μg./liver)	2756±266	2994±261	0·541	—	—	—
Units of enzyme/g. liver:						
G 6-P dehydrogenase, pH 7·6	120±27	177±39	0·269	139±14	130±8	0·596
6-PG dehydrogenase, pH 9·0	366±27	261±33	0·040	305±16	249±7	0·006
6-PG dehydrogenase, pH 7·6	—	—	—	142±6	110±3	<0·001
Total units of enzyme in liver:						
G 6-P dehydrogenase, pH 7·6	762±237	1345±331	0·191	907±101	935±66	0·820
6-PG dehydrogenase, pH 9·0	2187±233	1974±363	0·631	1906±104	1727±92	0·212
6-PG dehydrogenase, pH 7·6	—	—	—	931±67	793±45	0·101
Units of enzyme/mg. DNAP:						
G 6-P dehydrogenase, pH 7·6	651±114	954±223	0·264	—	—	—
6-PG dehydrogenase, pH 9·0	2089±329	1406±208	0·119	—	—	—

* Female hooded Norway rats injected subcutaneously with 400 μg. growth-hormone preparation 1 for 8 days.
† Female albino rats injected subcutaneously with 500 μg. growth-hormone preparation 2 for 10 days.

Thiouracil treatment

The dehydrogenase activities of the liver and voluntary muscle of thiouracil-treated and pair-fed control rats are given in Table 6. Although there is a significant decrease in the levels of activity of both dehydrogenases when expressed in units/g. liver, there is no significant change if the results are expressed in units/whole liver, on account of the significant increase in liver weight in the thiouracil-treated group of animals. The muscle-dehydrogenase levels are likewise unaffected.

Seasonal variations in dehydrogenase activities

Tables 1–6 show that although the dehydrogenase activities of the livers of the control rats show only small variations in any one experiment, the average dehydrogenase activities of the control groups of animals vary considerably in different experiments. This cannot be attributed to the paired-feeding technique and indicates seasonal variations in enzyme activities, as is shown in Fig. 2. The levels of activity of both dehydrogenases are considerably higher in summer than in winter.

Table 5. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in the livers of control and thyroxine-treated rats*

Results expressed as means ± S.E.M. For details, see Methods section.

	Control group	Thyroxine-treated group	*P*
No. of animals	6	6	—
Initial body wt. (g.)	175 ± 1	172 ± 1	—
Final body wt. (g.)	192 ± 3	167 ± 3	—
Liver wt. (g.)	7·52 ± 0·37	6·90 ± 0·24	—
Liver nitrogen (mg./g.)	35·8 ± 1·2	37·4 ± 0·4	0·258
Muscle nitrogen (mg./g)	35·2 ± 0·3	36·4 ± 0·3	0·019
Units of enzyme/g. liver:			
G 6-P dehydrogenase, pH 7·6	76 ± 10	151 ± 14	0·002
6-PG dehydrogenase, pH 9·0	264 ± 8	634 ± 32	<0·001
6-PG dehydrogenase, pH 7·6	119 ± 8	312 ± 18	<0·001
Total units of enzyme in liver:			
G 6-P dehydrogenase, pH 7·6	557 ± 61	1056 ± 128	0·006
6-PG dehydrogenase, pH 9·0	1985 ± 129	4394 ± 320	<0·001
6-PG dehydrogenase, pH 7·6	895 ± 66	2165 ± 172	<0·001
Units of enzyme/g. muscle*:			
G 6-P dehydrogenase, pH 7·6	7·5 ± 0·4	8·0 ± 0·4	0·431
6-PG dehydrogenase, pH 9·0	16·9 ± 0·5	16·6 ± 2·5	0·883

* Five rats in this group.

Table 6. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in the livers of control and thiouracil-treated rats*

Results expressed as means ± S.E.M. For details, see Methods section.

	Control group	Thiouracil-treated group	*P*
No. of animals	6	6	—
Initial body wt. (g.)	170 ± 1	169 ± 1	—
Final body wt. (g.)	204 ± 3	201 ± 5	—
Liver wt. (g.)	6·77 ± 0·23	8·09 ± 0·42	0·014
Thyroid wt. (mg.)	18 ± 1	53 ± 5	<0·001
Liver nitrogen (mg./g.)	37·4 ± 0·4	35·3 ± 0·9	0·055
Muscle nitrogen (mg./g.)	37·0 ± 1·4	34·2 ± 0·4	0·064
Units of enzyme/g. liver*:			
G 6-P dehydrogenase, pH 7·6	73 ± 11	44 ± 5	0·041
6-PG dehydrogenase, pH 9·0	196 ± 13	148 ± 7	0·011
Total units of enzyme in liver:			
G 6-P dehydrogenase, pH 7·6	479 ± 67	367 ± 61	0·251
6-PG dehydrogenase, pH 9·0	1293 ± 67	1210 ± 126	0·604
Units of enzyme/g. muscle:			
G 6-P dehydrogenase, pH 7·6	5·2 ± 0·4	4·3 ± 0·4	0·114
6-PG dehydrogenase, pH 9·0	11·1 ± 0·5	10·2 ± 0·6	0·272

* Five rats only in this group.

In vitro *experiments*

Neither G 6-P nor 6-PG dehydrogenase activities of rat-liver supernatants were affected by the addition of growth hormone, thyroxine or insulin in the concentrations employed and under the particular conditions of assay described in the experimental section.

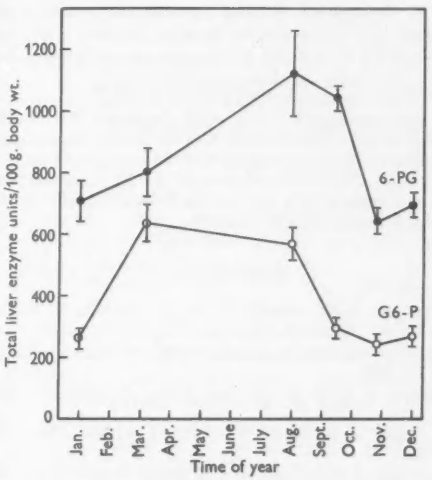

Fig. 2. Seasonal variations in the levels of activity of G 6-P and 6-PG dehydrogenases in rat liver. Adult male rats used. ○, G 6-P dehydrogenase, pH 7·6; ●, 6-PG dehydrogenase, pH 9·0. The vertical lines represent twice the S.E.M.

DISCUSSION

This investigation has shown that levels of activity of G 6-P and 6-PG dehydrogenases are significantly reduced in the livers of rats maintained on a restricted food intake for 8 days and also after 48 hr. starvation. The levels of both dehydrogenases are in addition markedly decreased in alloxan diabetes and increased as a result of thyroxine treatment. Although G 6-P dehydrogenase activity shows a tendency to increase as a result of long-term treatment with growth hormone, this is not statistically significant when expressed on a whole-liver basis, on account of the wide variations in the individual dehydrogenase activities in groups of female rats. The paired-feeding technique was employed in all these hormonal experiments in order to eliminate nutritional variations in dehydrogenase levels.

Alterations in enzyme contents of liver as a result of restricted food intake, low-protein diets and starvation have been reported frequently. In general, loss of enzyme activity has been shown to parallel a decrease in total liver protein (Potter & Klug, 1947; Seifter, Harkness, Rubin & Muntwyler, 1948; Miller, 1948, 1953), suggesting that liver-enzyme proteins enter into protein metabolism in a dynamic but more or less non-specific fashion. The alterations in levels of G 6-P and 6-PG dehydrogenases in whole liver as a result of restricted food intake and starvation appear to be no exception to this general rule.

The ubiquitous distribution of enzymes of the hexose monophosphate (HMP) oxidative pathway in plant and animal tissues and the high levels of activity of G 6-P and 6-PG dehydrogenases in certain mammalian tissues (Glock & McLean, 1954) suggest that there is active competition between the glycolytic and HMP oxidative pathways for available G 6-P. Since in the steady state of intracellular metabolism the concentration of G 6-P is very low, the relative affinities of G 6-P for competing enzymes of the two alternative pathways must play a decisive part in determining its ultimate fate under physiological conditions. If the assumption is made that hexose phosphate isomerase is never a limiting factor, published values for the Michaelis constants (K_m) of 6-phosphofructokinase and G 6-P dehydrogenase, the first enzyme steps in the glycolytic and HMP oxidative pathways respectively, indicate that the latter pathway can indeed actively compete for G 6-P. Thus the K_m (F 6-P) of rabbit-muscle phosphofructokinase is 1×10^{-4} M (see Stumpf, 1954) and the K_m (G 6-P) for G 6-P dehydrogenase of rat liver is $1{\cdot}3 \times 10^{-5}$ M (Glock & McLean, 1953). Since G 6-P is the common substrate for both metabolic pathways, hormonal effects at the hexokinase level would be expected to affect both pathways in a similar fashion. Indirect evidence, particularly from experiments on the utilization of [^{14}C]glucose and [^{14}C]fructose by rat-liver slices, indicates that there is in fact a metabolic block at the glucokinase stage both in experimental diabetes and as a result of starvation for 48 hr. or longer (see Chernick & Chaikoff, 1951; Chernick, Chaikoff & Abraham, 1951; Wyshak & Chaikoff, 1953; Renold, Teng, Nesbett & Hastings, 1953; Renold, Hastings & Nesbett, 1954). The existence of such a block could account for the lowered levels of activity of G 6-P and 6-PG dehydrogenases observed under similar conditions in the present investigation. No data are available on the effects of growth hormone and thyroxine on liver hexokinase, although thyroxine treatment has been shown to increase muscle-hexokinase activity (Smith & Williams-Ashman, 1951).

The most important contributions to an evaluation of alternative pathways have come from isotope studies with tissue slices in which the

yields of $^{14}CO_2$ from (6-^{14}C)glucose and (1-^{14}C)-glucose are compared. Preferential conversion of C_1 of glucose into CO_2 indicates the occurrence of a non-glycolytic pathway. Results obtained by Agranoff, Brady & Colodzin (1954) are in support of active competition between the glycolytic and non-glycolytic pathways for G 6-P, since, in liver slices from rats fed on a high-glucose diet, there was an approximately threefold increase in $^{14}CO_2$ from both (1-^{14}C)glucose and (6-^{14}C)glucose without an increase in the quotient (yield of $^{14}CO_2$ from C_6)/(yield of $^{14}CO_2$ from C_1), this quotient remaining approximately constant at 0·33–0·29. This work also corroborates our findings about the reduced activity of the HMP oxidative pathway after 48 hr. starvation. The non-glycolytic pathway was still further reduced after 72 hr. starvation, the C_6/C_1 quotient rising to 0·7. Although Katz, Abraham, Hill & Chaikoff (1954) consider that not more than 10% of the glucose utilization by liver slices can proceed by non-glycolytic pathways, the work of Agranoff *et al.* (1954) and of Bloom & Stetten (1955) indicates a much more active participation of non-glycolytic routes. In diaphragm, however, glucose metabolism proceeds probably exclusively via glycolysis (Bloom & Stetten, 1953; Agranoff *et al.* 1954), which is in accord with the very low levels of activity of enzymes of the HMP oxidative pathway in voluntary muscle (Glock & McLean, 1954).

It is of some interest that the steady decline in total dehydrogenase activities in the livers of rats on a restricted food intake parallels an accompanying decrease in total liver RNA levels. Lowered dehydrogenase levels in alloxan diabetes are accompanied by a similar reduction in the total RNA content (cf. Thomson *et al.* 1953). Although these changes in dehydrogenase and RNA levels are probably non-specific manifestations of alterations in protein synthesis in general, it is attractive to conjecture that RNA levels parallel dehydrogenase activities, since the HMP oxidative pathway may serve as a source of ribose for RNA synthesis.

SUMMARY

1. Total levels of activity of both glucose 6-phosphate (G 6-P) and 6-phosphogluconate (6-PG) dehydrogenases are significantly reduced in the livers of rats maintained on restricted food intakes for 8 days and also after 48 hr. starvation.

2. Concentrations of both dehydrogenases are markedly reduced in alloxan diabetes, total liver activities being only approximately one-third of the control values.

3. Thyroxine treatment results in an approximately twofold increase in the levels of activity of both dehydrogenases, whereas thiouracil treatment, although decreasing enzyme activities/g. liver, does not significantly affect total liver values.

4. Although long-term treatment with crystalline growth hormone causes some increase in G 6-P dehydrogenase activity/g. liver, total liver-enzyme activities are not significantly affected.

5. Liver-dehydrogenase concentrations show seasonal variations and are significantly higher in summer than in winter.

6. Crystalline insulin, crystalline growth hormone and DL-thyroxine have no *in vitro* effect on either G 6-P or 6-PG dehydrogenase activity under the particular conditions employed.

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Effects of Hormones on Levels of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Liver and Diaphragm

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Variations in tissue levels of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), as well as the relative proportions of oxidized and reduced coenzymes, presumably play an important part in the regulation of alternative pathways of carbohydrate metabolism. For this reason, the methods described by Glock & McLean (1955*a*) for the separate determination of oxidized and reduced DPN and TPN in animal tissues have been applied to the estimation of DPN^+, DPNH, TPN^+ and TPNH in the livers and voluntary muscles of rats subjected to various dietary and hormonal procedures. The present paper deals with the effects of starvation, alloxan diabetes and treatment with growth hormone, thyroxine and thiouracil. Changes in the total DPN content of liver, as well as in the proportion of oxidized to reduced coenzyme, have recently been reported by Helmreich, Holzer, Lamprecht & Goldschmidt (1954) in alloxan diabetes and in starvation.

METHODS

Treatment of animals. Young adult albino rats were used. The dietary procedure, production of alloxan diabetes, conditions for studying the effects of starvation and treatment with growth hormone, thyroxine and thiouracil were the same as described in the preceding paper (Glock & McLean, 1955*b*).

Estimation of coenzymes. The preparation of substrates and enzymes was carried out as described previously (Glock & McLean, 1955*a*), except for cytochrome *c*, which was a commercial preparation (Sigma Chemical Co.). Oxidized and reduced coenzymes were determined, respectively, in neutralized acid and alkaline tissue extracts (Glock & McLean, 1955*a*).

RESULTS

Alloxan diabetes and starvation

The contents of oxidized and reduced DPN and TPN in the liver and diaphragm of alloxan-diabetic and pair-fed control rats are given in Table 1. Liver values after 24 and 48 hr. starvation are shown in Table 2. In the case of the alloxan-diabetic rats, there was a significant fall in the concentration of oxidized DPN/g. liver with a corresponding decrease in the DPN^+/DPNH quotient. Although the total DPN (DPN^+ + DPNH) content was unaltered on a g. liver basis there was a significant decrease on a whole-liver basis. There were much less striking changes in the TPN contents. The total TPN (principally TPNH)/g. liver was unaffected, but again total TPN/whole liver was significantly reduced. In diaphragm there was some rise in the concentration of oxidized DPN/g. muscle, with a small but significant increase in the DPN^+/DPNH quotient, the total DPN concentration being unaffected. When expressed on a whole-diaphragm basis, there was a small but significant decrease in both DPNH and total DPN (DPN^+ + DPNH) contents. The total TPN content of diaphragm is too small to warrant further investigation.

The liver values of rats starved for 24 and 48 hr. (see Table 2) indicate that, as in alloxan diabetes, there is a marked fall in the DPN^+/DPNH quotient when compared with the average quotient of control rats (Table 1). In addition, there was a marked reduction in the concentration of both oxidized and reduced DPN when the starvation period was extended to 48 hr.

Growth-hormone treatment

Values for the DPN^+, DPNH, TPN^+ and TPNH contents of the livers of control and pair-fed growth-hormone-treated female rats are given in Table 3. As in previous experiments (Glock & McLean, 1955*b*), two different growth-hormone preparations were used, GH 1 and GH 2. Only GH 1 produced any significant changes in coenzyme levels, which can presumably be attributed to the fact that this preparation was more effective in promoting growth, particularly of the diaphragm, than GH 2. It is of interest that GH 1 was also much more effective than GH 2 in causing an increase in the G 6-P dehydrogenase activity of liver (Glock & McLean, 1955*b*).

Table 1. *Levels of oxidized and reduced coenzymes in liver and diaphragm of control and alloxan-diabetic rats*

Results expressed as means ± S.E.M. Duration of diabetes, 10 days.

	Control group	Alloxan-treated group	*P*
No. of animals	5	5	—
Initial body wt. (g.)	201 ± 5	201 ± 6	—
Final body wt. (g.)	230 ± 6	159 ± 10	—
Liver wt. (g.)	10·11 ± 0·48	7·33 ± 0·59	0·006
Diaphragm wt. (mg.)	380 ± 8	290 ± 17	0·001
Average daily sugar excretion (g.)	—	4·3 ± 0·5	—
Liver coenzymes (μg./g.)			
DPN^+	407 ± 13	324 ± 18	0·007
DPNH	161 ± 17	217 ± 23	0·086
$DPN^+ + DPNH$	568 ± 27	541 ± 28	0·504
$DPN^+/DPNH$	2·70 ± 0·31	1·53 ± 0·15	0·011
TPN^+	10 ± 1	8 ± 1	0·115
TPNH	148 ± 5	159 ± 6	0·195
Total liver coenzymes (μg.):			
DPN^+	4140 ± 330	2387 ± 251	0·003
DPNH	1651 ± 247	1565 ± 129	0·772
$DPN^+ + DPNH$	5791 ± 541	3952 ± 315	0·019
TPN^+	104 ± 11	58 ± 10	0·017
TPNH	1496 ± 67	1153 ± 73	0·009
Diaphragm coenzymes (μg./g.):			
DPN^+	263 ± 7	307 ± 17	0·050
DPNH	164 ± 25	143 ± 9	0·119
$DPN^+ + DPNH$	427 ± 12	450 ± 20	0·345
$DPN^+/DPNH$	1·61 ± 0·08	2·18 ± 0·17	0·016
TPN^+	3 ± 1	2 ± 1	0·360
TPNH	12 ± 1	13 ± 2	0·847
Total diaphragm coenzymes (μg.):			
DPN^+	100 ± 2	89 ± 6	0·132
DPNH	63 ± 3	42 ± 4	0·005
$DPN^+ + DPNH$	163 ± 5	131 ± 9	0·017

Table 2. *Effect of starvation on the levels of oxidized and reduced coenzymes in rat liver*

Results expressed as means ± S.E.M.

	Starved for 24 hr.	Starved for 48 hr.	*P*
No. of animals	6	6	—
Initial body wt. (g.)	182 ± 1	179 ± 2	—
Final body wt. (g.)	170 ± 3	153 ± 2	—
Liver wt. (g.)	5·50 ± 0·06	5·03 ± 0·17	0·026
Liver coenzymes (μg./g.):			
DPN^+	333 ± 24	212 ± 26	0·008
DPNH	202 ± 16	121 ± 9	0·002
$DPN^+ + DPNH$	535 ± 35	333 ± 34	0·003
$DPN^+/DPNH$	1·67 ± 0·12	1·74 ± 0·14	0·761
TPN^+	20 ± 6	<2	—
TPNH	308 ± 34	308 ± 24	—
Total liver coenzymes (μg.):			
DPN^+	1825 ± 118	1086 ± 184	0·007
DPNH	1111 ± 84	617 ± 66	0·001
$DPN^+ + DPNH$	2935 ± 175	1702 ± 246	0·002
TPN^+	109 ± 29	<10	—
TPNH	1689 ± 184	1527 ± 88	0·648

As Table 3 shows, GH 1 produced a marked increase in DPN^+, DPNH and total DPN (DPN^+ + DPNH) contents/g. liver, all these increases being accentuated when expressed on a whole-liver basis. The concentration of TPNH/g. liver was not affected, but on account of the increase in liver size resulting from growth-hormone treatment, TPNH/whole liver was increased. No significant alterations were found in the coenzyme contents of diaphragm.

a whole-liver basis. The most marked effect was on the total TPN (i.e. principally TPNH) content of liver, which was strikingly decreased both on a unit-weight and on a whole-liver basis. No significant alterations in coenzyme concentrations were found in diaphragm.

Results for thiouracil-treated and pair-fed control rats are given in Table 5. Neither liver nor diaphragm showed any marked alterations in DPN or TPN contents.

Table 3. *Levels of oxidized and reduced coenzymes in liver and diaphragm of control and growth-hormone-treated rats*

Results expressed as means ± S.E.M.

	Control group	Growth-hormone-treated group*	*P*	Control group	Growth-hormone-treated group†	*P*
No. of animals	5	5	—	11	11	—
Initial body wt. (g).	158±4	157±4	—	186±6	184±5	—
Final body wt. (g.)	164±7	180±4	—	192±5	205±4	—
Liver wt. (g.)	5·96±0·51	7·50±0·81	0·148	6·53±0·27	7·16±0·26	0·105
Diaphragm wt. (mg.)	403±15	492±27	0·023	366±22	394±22	0·379
Liver coenzymes (μg./g.):						
DPN^+	330±9	421±38	0·051	289±17	326±10	0·069
DPNH	133±8	206±27	0·035	136±12	118±9	0·264
DPN^+ + DPNH	463±17	627±49	0·014	425±20	444±16	0·472
DPN^+/DPNH	2·49±0·09	2·24±0·43	0·565	2·30±0·22	2·91±0·22	0·068
TPN^+	10±1	6±2	0·059	3±1	2	—
TPNH	185±16	199±18	0·565	193±10	196±9	0·828
Total liver coenzymes (μg.):						
DPN^+	1962±181	3157±453	0·040	1897±147	2332±110	0·028
DPNH	788±69	1465±111	0·001	871±64	845±71	0·761
DPN^+ + DPNH	2749±247	4622±427	0·005	2767±157	3164±167	0·099
TPN^+	56±7	38±10	0·172	19±6	<14	—
TPNH	1104±142	1449±91	0·076	1268±96	1403±81	0·284
Diaphragm coenzymes (μg./g.):						
DPN^+	383‡	366‡	—	257±16	252±15	0·822
DPNH	271	236	—	97±16	105±19	0·755
DPN^+ + DPNH	656	602	—	354±25	357±16	0·922
DPN^+/DPNH	1·42	1·55	—	2·96±0·35	2·88±0·44	0·890
TPN^+	3	3	—	<2	<2	—
TPNH	18	15	—	14±1	11±3	0·608

* Female hooded Norway rats injected daily subcutaneously with 400 μg. growth-hormone preparation 1 for 8 days.
† Female albino rats injected daily subcutaneously with 500 μg. growth-hormone preparation 2 for 10 days. (For description of growth-hormone preparations see Glock & McLean, 1955*b*.)
‡ Values for pooled diaphragm extracts.

The total liver-DPN values in this experiment were considerably lower than in any of the others. This may, perhaps, be attributed to the fact that this was the only experiment employing female rats.

Thyroxine and thiouracil treatment

The coenzyme contents of the liver and diaphragm of control and pair-fed thyroxine-treated rats are given in Table 4. Thyroxine treatment resulted in a fall in the concentration of oxidized DPN/g. liver and a marked decrease in both oxidized DPN and total DPN (DPN^+ + DPNH) on

DISCUSSION

Both starvation and experimental diabetes result in a marked inhibition of fatty acid synthesis. In the former case, this can be restored to normal by glucose only, whereas both glucose and insulin are necessary to reverse the diabetic condition (see Chaikoff, 1951–52; Stadie, 1954). Lipogenesis is dependent on the supply of acetyl coenzyme A and DPNH, derived from the breakdown of carbohydrate. For lipogenesis to proceed at a significant rate, it is necessary to maintain a high concentration of both these substrates in order to shift the

Table 4. *Levels of oxidized and reduced coenzymes in liver and diaphragm of control and thyroxine-treated rats*

Results expressed as means ± S.E.M.

	Control group	Thyroxine-treated group	*P*
No. of animals	6	6	—
Initial body wt. (g.)	170 ± 1	172 ± 2	—
Final body wt. (g.)	207 ± 3	170 ± 4	—
Liver wt. (g.)	8·57 ± 0·09	7·26 ± 0·21	<0·001
Diaphragm wt. (mg.)	350 ± 13	288 ± 9	0·003
Liver coenzymes (μg./g.):			
DPN^+	446 ± 23	393 ± 7	0·057
DPNH	158 ± 9	164 ± 10	0·628
$DPN^+ + DPNH$	604 ± 22	557 ± 16	0·113
$DPN^+/DPNH$	2·86 ± 0·25	2·43 ± 0·12	0·150
TPN^+	4 ± 1	<2	—
TPNH	206 ± 8	155 ± 10	0·003
Total liver coenzymes (μg.):			
DPN^+	3818 ± 195	2856 ± 111	0·001
DPNH	1357 ± 81	1193 ± 85	0·192
$DPN^+ + DPNH$	5176 ± 188	4049 ± 185	0·001
TPN^+	30 ± 10	<14	—
TPNH	1762 ± 73	1119 ± 68	<0·001
Diaphragm coenzymes (μg./g.):			
DPN^+	301 ± 26	354 ± 25	0·172
DPNH	109 ± 13	102 ± 10	0·631
$DPN^+ + DPNH$	424 ± 27	456 ± 33	0·504
$DPN^+/DPNH$	3·06 ± 0·57	3·57 ± 0·22	0·347
TPN^+ (pooled values)	<2	<2	—
TPNH (pooled values)	14	15	—
Total diaphragm coenzymes (μg.):			
DPN^+	104 ± 8	102 ± 7	0·845
DPNH	36 ± 4	29 ± 3	0·144
$DPN^+ + DPNH$	142 ± 4	131 ± 9	0·347

Table 5. *Levels of oxidized and reduced coenzymes in liver and diaphragm of control and thiouracil-treated rats*

Results expressed as means ± S.E.M.

	Control group	Thiouracil-treated group	*P*
No. of animals	6	6	—
Initial body wt. (g.)	163 ± 3	163 ± 2	—
Final body wt. (g.)	233 ± 5	206 ± 4	—
Liver wt. (g.)	8·27 ± 0·22	9·04 ± 0·23	0·037
Diaphragm wt. (mg.)	403 ± 20	377 ± 12	0·297
Liver coenzymes (μg./g.):			
DPN^+	370 ± 19	363 ± 13	0·770
DPNH	204 ± 11	211 ± 17	0·698
$DPN^+ + DPNH$	574 ± 29	574 ± 27	—
$DPN^+/DPNH$	1·82 ± 0·07	1·75 ± 0·10	0·562
TPN^+	8 ± 3	14 ± 2	0·054
TPNH	309 ± 22	311 ± 16	0·922
Total liver coenzymes (μg.)·			
DPN^+	3044 ± 118	3292 ± 190	0·297
DPNH	1680 ± 77	1917 ± 174	0·244
$DPN^+ + DPNH$	4724 ± 179	5209 ± 340	0·236
TPN^+	63 ± 8	128 ± 5	0·003
TPNH	2546 ± 164	2809 ± 145	0·273
Diaphragm coenzymes (μg./g.):			
DPN^+	217 ± 11	192 ± 11	0·141
DPNH	103 ± 3	109 ± 5	0·341
$DPN^+ + DPNH$	319 ± 14	302 ± 14	0·389
$DPN^+/DPNH$	2·11 ± 0·07	1·74 ± 0·10	0·012
TPN^+ (pooled values)	2	10	—
TPNH (pooled values)	13	40	—
Total diaphragm coenzymes (μg.):			
DPN^+	87 ± 4	72 ± 4	0·023
DPNH	41 ± 2	41 ± 1	—
$DPN^+ + DPNH$	128 ± 5	113 ± 5	0·048

equilibrium of β-ketothiolase and β-ketoreductase in the direction of synthesis (see Lynen, 1954; Green, 1954). In diabetes and starvation, where the metabolism of carbohydrate is depressed and the synthesis of fatty acids restricted, an alteration in the steady-state level of DPN^+/DPNH would be expected. A change in the DPN^+/DPNH quotient has indeed been found in rat livers in starvation and diabetes both by Helmreich *et al.* (1954) and by us. The former workers obtained an increase in the DPN^+/DPNH quotient due chiefly, but not entirely, to a decrease in the concentration of DPNH, whereas we have found a decreased DPN^+/DPNH quotient resulting from a fall in the concentration of DPN^+. It is difficult to account for these diametrically opposed results. The only obvious differences in experimental procedures are that Helmreich *et al.* (1954) did not employ the paired-feeding technique, the effects of starvation were studied after as long as 72–120 hr., when they were no longer reversible by glucose, and the degree of diabetes was probably more severe since several rats were killed in diabetic coma. These workers consider that ketone-body accumulation and decreased DPNH concentrations in starvation and diabetes are the outcome of deranged glycolysis and that in both conditions low concentrations of DPNH are responsible for restricted fatty acid synthesis. Our results, on the other hand, could be explained on the basis that lipogenesis is known to be more strikingly reduced than the glycolytic breakdown of glucose in both diabetic and fasting rats (see Chaikoff, 1951–52; Stadie, 1954) and that fatty acid oxidation proceeds normally in diabetic liver (see Green, 1954). Thus two of the enzyme systems causing reduction of DPN^+ to DPNH are relatively unimpaired, whereas lipogenesis, a synthetic process requiring DPNH, is strongly inhibited. This would result in an accumulation of DPNH and a reduction in the level of oxidized DPN. It is of interest that the DPN^+/DPNH quotient is unchanged in the livers of rats after growth-hormone treatment at a time when there is increased fat catabolism, as shown by changes in R.Q. and body composition (Greenbaum, 1953; Young, 1953).

It is now generally believed that the action of thyroxine in controlling tissue respiration is located not at the level of the individual respiratory enzymes but in the mitochondria, where respiration is coupled to the production of high-energy phosphate bonds. Under specified conditions, both thyroxine and triiodothyronine can uncouple oxidative phosphorylation in mitochondrial preparations *in vitro* (see Maley & Lardy, 1953; Lardy & Maley, 1954) and the *in vitro* incorporation of ^{32}P into mitochondrial ATP has also been shown to be depressed (Martius & Hess, 1951). Moreover, reduced P:O ratios have been reported in mitochondrial preparations from hyperthyroid animals by two groups of investigators (Lardy & Feldott, 1951; Hoch & Lipmann, 1953). It should be emphasized, however, that these effects can be demonstrated only under certain specified conditions using large unphysiological doses of thyroxine (see Du Toit, 1952). Reduced mitochondrial synthesis of ATP in hyperthyroidism could account for the reduced DPN and TPN concentration in the livers of rats after thyroxine treatment. The more or less uniform intracellular distribution of [^{131}I]-thyroxine between mitochondrial, nuclear and supernatant liver fractions (Lipner, Klitgaard, Barker & Winnick, 1952; Crane, 1952) would favour any active participation of this hormone in the mitochondrial synthesis of ATP and in nuclear coenzyme synthesis. An effect of thyroxine on coenzyme synthesis is, however, purely hypothetical. It is of interest that whereas thyroxine results in a marked reduction in total DPN and TPN concentrations in liver, diaphragm coenzyme levels are not appreciably affected. This can perhaps be attributed to the fact that injected [^{131}I]thyroxine is preferentially concentrated in the liver (Albert & Keating, 1952).

Marked variations in total DPN and TPN tissue concentrations, such as occur in rat livers as a result of thyroxine and growth-hormone treatments, are presumably due to direct or indirect effects of these hormones on coenzyme synthesis. It would be interesting to see whether hormones have any *in vitro* effects on coenzyme synthesis in isolated nuclei.

SUMMARY

1. Oxidized and reduced DPN and TPN contents of liver and diaphragm have been determined in control, diabetic and fasting rats and in rats subjected to treatment with growth hormone, thyroxine and thiouracil.

2. Total DPN (DPN^+ + DPNH) contents of liver were markedly reduced in alloxan diabetes and after 48 hr. starvation. A decrease was also found after thyroxine treatment. Striking increases in total DPN contents of liver were, on the contrary, found after treatment with one crystalline growth-hormone preparation but not with a less active preparation.

3. Total liver TPN (principally TPNH) contents were strikingly reduced as a result of thyroxine treatment, although thiouracil produced no significant changes.

4. The DPN^+/DPNH quotient of rat liver was significantly reduced in alloxan diabetes.

5. Alterations in diaphragm coenzyme contents were less striking than those found in liver.

6. An attempt has been made to account for the low $DPN^+/DPNH$ quotient found in diabetic liver, and it has been tentatively suggested that hormones may directly or indirectly regulate the synthesis of both DPN and TPN.

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The Metabolism of Phospholipids in the Lactating Mammary Gland of the Rabbit

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The experiments to be described were undertaken as part of a study to determine the role of blood lipids in the metabolism of the lactating mammary gland. Contrary to the views formerly held (e.g. Meigs, Blatherwick & Cary, 1919) that plasma phospholipids are the precursors of the fatty acids of milk fat, arteriovenous studies subsequently indicated that no uptake of phospholipids into the lactating bovine gland apparently takes place (Blackwood, 1934) and that no fraction of blood lipids except glycerides and possibly cholesteryl esters are absorbed from the blood stream by the ruminant udder (Lintzel, 1934; Graham, Jones & Kay, 1936; Maynard, McCay, Ellis, Hodson & Davis, 1938; Shaw & Petersen, 1940; Voris, Ellis & Maynard, 1940). Aten & Hevesy (1938), in one of the first studies of lactation involving radioisotopes, showed that phospholipids are synthesized in the lactating udder of the goat, but contended that their results were not consistent with the view that plasma phospholipids are degraded in the mammary gland.

It will be shown, however, that in the lactating rabbit, phospholipids are not only synthesized in the mammary gland, but are also absorbed from the plasma and degraded in the gland to a significant extent. Though this study was primarily concerned with the metabolism of lactating mammary gland tissue, the opportunity was taken to examine the corresponding phosphorus-containing fractions of the livers of the animals under investigation. A preliminary report of part of this work has already appeared (Garton & Popják, 1952).

EXPERIMENTAL

Administration of ^{32}P-labelled inorganic phosphate to lactating rabbits

Two lactating Chinchilla rabbits were injected intravenously with 57·5 μc (rabbit 1) and 66 μc (rabbit 2) of [^{32}P]orthophosphate contained in 0·9% NaCl. Two hours later rabbit 1 was anaesthetized with nembutal–ether and mammary-gland tissue removed, together with a piece of liver. The tissue samples were carefully blotted with filter paper, cut into small pieces and preserved in a freezing mixture of acetone and solid CO_2 below $-70°$. Rabbit 2 was similarly treated, except that before tissue samples were taken the animal was given a subcutaneous injection of about 2 i.u. oxytocin (Pitocin, Parke, Davis and Co. Ltd., Hounslow, Middlesex) and milked, the suction technique described by Popják, Hunter & French (1953) being used to yield about 20 ml. milk; in addition, samples of mammary gland and liver were taken and blood was withdrawn from the aorta into heparinized receivers and immediately centrifuged to obtain plasma.

Preparation of plasma containing labelled phospholipids

To render them lipaemic, two adult Chinchilla male rabbits were each given, by stomach tube, a daily administration for 18 days of 1 g. amorphous cholesterol suspended in water (cf. Popják, 1946). Each animal was then given two successive daily intravenous injections each of approximately 1·0 mc [^{32}P]orthophosphate in warm 0·9% saline. One day later the animals were exsanguinated and the pooled plasma was then treated essentially as described by Popják & Beeckmans (1950) for the removal of inorganic phosphate and other small molecules by dialysis. The preparation of labelled plasma was carried out at different times on three pairs of animals.

Administration of plasma containing labelled phospholipids

Blood (10–20 ml.) was withdrawn from a marginal ear vein of each of four lactating Chinchilla rabbits immediately before the intravenous injection of labelled plasma, the properties of which are shown in Table 1.

Table 1. *Properties of dialysed rabbit plasma containing* ^{32}P*-labelled phospholipids used for injection into lactating rabbits*

^{32}P radioactivity associated with	Counts/min./ml.*	Percentage of total activity
Plasma injected into rabbit 3		
Phospholipids	2559	63·3
Acid-soluble P	136	3·4
Phosphoprotein	1345†	33·3
Total ^{32}P content	4040	
Plasma injected into rabbit 4		
Phospholipids	4380	79·2
Acid-soluble P	44	0·8
Phosphoprotein	1107†	20·0
Total ^{32}P content	5531	
Plasma injected into rabbits 5 and 6		
Phospholipids	4012	74·7
Acid-soluble P	63	1·2
Phosphoprotein	1295†	24·1
Total ^{32}P content	5370	

* On day of injection. † By difference.

Rabbit 3 received 20 ml. plasma, rabbit 4 received 10 ml., and rabbits 5 and 6 each received 15 ml. The plasma was warmed to 37° before injection. Approximately 2 hr. later the animals were anaesthetized with nembutal–ether and blood was withdrawn from the aorta or taken by heart puncture; pieces of mammary gland and liver were then taken and treated, before analysis, as described above. Milk samples (10–20 ml.) were also obtained by suction from rabbits 4–6 before any surgical operations were carried out.

Treatment of tissue samples

Mammary gland and liver. The samples were removed from the freezing mixture (usually within a few hours) and ground to powder in a mortar which had been pre-cooled with an acetone–solid CO_2 mixture. The powdered tissue (about 10 g.) was then divided into two parts, each of which was weighed to the nearest 100 mg. One part was taken for the extraction of phospholipids, as described by Popják & Beeckmans (1950), and the other was extracted with ice-cold 10% (w/v) trichloroacetic acid to yield the acid-soluble P compounds, which were separated into fractions giving soluble and insoluble Ba salts, according to Kaplan & Greenberg (1944). The salts yielding insoluble Ba salts were dissolved in 5 ml. 0·1 N-HCl and centrifuged to remove a trace of insoluble matter; inorganic phosphate in the supernatant liquid was then precipitated as NH_4MgPO_4 in the usual way. The P compounds forming soluble Ba salts were heated with N-NaOH on a boiling-water bath for 0·5 hr. and the phosphate liberated from the alkali-labile compounds was removed. The organic phosphate which was resistant to alkaline hydrolysis was taken to represent glycerophosphate (cf. Kaplan & Greenberg, 1944; Popják & Muir, 1950). By wet-ashing (Kjeldahl), the glycerophosphate and phospholipid fractions were converted into inorganic phosphate, which was subsequently precipitated as NH_4MgPO_4. The NH_4MgPO_4 obtained from all three P-containing fractions (inorganic, glycerophosphate and phospholipid) was dissolved in a few drops of 0·1 N-HCl and diluted to a known volume (usually 25 ml.) with distilled water.

Plasma and milk. Milk (usually 10 ml.) or plasma (5, 10 or 20 ml.) was extracted with a 20-fold excess of boiling ethanol–ether (3:1, by vol.) and the lipid extracts were treated as described by Popják & Beeckmans (1950) for the separation of phospholipids and their conversion into inorganic P, which was precipitated as NH_4MgPO_4, dissolved in a few drops of 0·1 N-HCl and diluted to a known volume.

Estimation of inorganic phosphate and assay of ^{32}P

Inorganic P was determined by the method of Allen (1940) or Berenblum & Chain (1938). The radioactivity of the solutions was measured in a 'liquid' Geiger–Müller counter (Veall, 1948); the counts recorded were corrected for background counts and counts lost due to the 'dead' time of the circuit. Since the ^{32}P assays in each experiment were performed on different days, the activities recorded were corrected for radioactive decay to give the counts on the day the animals were injected with labelled phosphate or phospholipids. Specific radioactivities are expressed as counts/min./mg. P.

RESULTS AND DISCUSSION

In Table 2 are shown the specific radioactivities of the various P fractions from the tissues of the two lactating rabbits which were injected with [^{32}P]-orthophosphate.

The results obtained with rabbit 2 present a similar picture to that found by Aten & Hevesy (1938) in the lactating goat. The specific radioactivity of the mammary gland (and milk) phospholipid P is greater than the specific radioactivity of the plasma-phospholipid P, and so phospholipids must have been elaborated *de novo* in the gland and secreted into the milk. The specific radioactivities

Table 2. *Specific radioactivities of inorganic, glycerophosphate and phospholipid phosphorus in tissues of lactating rabbits after the intravenous injection of* [^{32}P]*orthophosphate*

Rabbit 1 received 57·5 μc and rabbit 2, 66 μc; specific radioactivities, expressed as counts/min./mg. P, were determined 2 hr. after injection.

	Rabbit 1. Specific radioactivity of			Rabbit 2. Specific radioactivity of		
	Inorganic P	Glycero-phosphate P	Phospholipid P	Inorganic P	Glycero-phosphate P	Phospholipid P
Mammary gland	12 640	2725	281	5 260	1895	262
Milk	n.d.*	n.d.	n.d.	7 420	n.d.	107
Plasma	n.d.	n.d.	n.d.	10 230	n.d.	99
Liver	23 850	3600	253	12 260	5275	636

* n.d. = not determined.

Table 3. *Specific radioactivity (counts/min./mg. P) of inorganic, glycerophosphate and phospholipid phosphorus in various tissues 2 hr. after injection of* [^{32}P]*phospholipids into lactating rabbits*

	Specific radioactivity of		
	Inorganic P	Glycero-phosphate P	Phospho-lipid P
	Rabbit 3		
Mammary gland	12	39	157
Plasma	n.d.*	n.d.	3640
Liver	Inactive	48	190
	Rabbit 4		
Mammary gland	3	48	85
Plasma	n.d.	n.d.	2629
Liver	18	49	127
Milk	n.d.	n.d.	Inactive
	Rabbit 5		
Mammary gland	7	79	114
Plasma	n.d.	n.d.	3753
Liver	133	79	357
Milk	n.d.	n.d.	Inactive
	Rabbit 6		
Mammary gland	3	22	99
Plasma	n.d.	n.d.	3406
Liver	7	39	134
Milk	n.d.	n.d.	Inactive

* n.d. = not determined.

of the mammary gland and liver phospholipid P of rabbit 1 are 10·3 and 7·0% respectively of the specific radioactivity of the corresponding precursor glycerophosphate P (cf. Popják & Muir, 1950); for rabbit 2 the corresponding figures are 13·8 and 12·05% respectively.

The specific radioactivities of the various tissue P compounds 2 hr. after the intravenous injection of the respective plasma preparations are given in Table 3. No activity was detected in the phospholipid P of the milk of rabbits 4–6. In the preliminary report (Garton & Popják, 1952) it is stated that radioactive phospholipids were isolated from the milk of a lactating animal which received ^{32}P-labelled phospholipids by injection (rabbit 3 of this paper). Contamination of the milk with blood was suspected, however, since most of the milk from this animal was collected from ducts during dissection of the mammary gland. This suspicion was confirmed by the failure to find any activity in the milk phospholipids of the animals which were subsequently treated in a similar manner except that they were milked by application of suction to the teats.

It has been shown (Popják & Muir, 1950) that the P precursor and first degradation product of lecithin and kephalin in the liver and placenta of the rabbit is glycerophosphate. The findings in the present work, that the specific radioactivities of the P fractions of mammary gland (and liver) were in the descending order phospholipid P, glycerophosphate P, inorganic P after the intravenous injection of labelled phospholipids, indicate that phospholipids were taken up from the plasma and broken down in these organs.

The specific radioactivity of the inorganic P of the liver of rabbit 5 was greater than that of the glycerophosphate P, and it seems likely that this finding is attributable to a rapid hepatic degradation, by this particular animal, of absorbed [^{32}P]-phosphoprotein in addition to glycerophosphate; a similar case was reported by Popják & Muir (1950).

The concentrations of glycerophosphate P and phospholipid P in the mammary-gland tissue of the animals which were injected with ^{32}P-labelled phospholipids are shown in Table 4.

Table 4. *Concentration of glycerophosphate* P *and phospholipid* P *in the mammary-gland tissue of lactating rabbits*

	Concn. (mg./100 g. of frozen tissue)	
Rabbit	Glycerophosphate P	Phospholipid P
3	66	83
4	22	47
5	19	57
6	Lost	54

Table 5. *Proportions of phospholipids derived from the plasma phospholipids and degraded in the lactating mammary glands of rabbits 2 hr. after the injection of* [^{32}P]*phospholipids*

Rabbit	Percentage of phospholipids derived from plasma*	Percentage of phospholipids degraded†
3	4·3	19·8
4	3·2	26·4
5	3·0	23·1
6	2·9	—

* Derived from specific radioactivities given in Table 3 by the expression:

$$\frac{\text{Specific radioactivity of mammary-gland phospholipid P} \times 100}{\text{Specific radioactivity of plasma phospholipid P}}.$$

† Derived as explained in text.

The amount of phospholipid degraded is derived on the assumption that the specific radioactivity of the glycerophosphate P produced by the breakdown of phospholipid is the same as the specific radioactivity of the phospholipid P. This assumption is supported by the findings of Taurog, Entenman & Chaikoff (1944) that plasma phospholipids consist chiefly of lecithin and a little sphingomyelin, and so we are here concerned with the uptake from the plasma and turnover of essentially only one molecular species. Thus the amount (mg./100 g.) of glycerophosphate P derived from phospholipid P in 2 hr. is given by the expression:

$$\frac{\text{[Specific radioactivity of glycerophosphate P] [Concn. (mg./100 g.) of glycerophosphate P]}}{\text{Specific radioactivity of phospholipid P}}.$$

This amount, expressed as a percentage of the concentration of phospholipid P in the respective mammary-gland tissues, gives the extent of phospholipid degradation, as shown in Table 5.

About 20% of the phospholipids present in the mammary gland were broken down in 2 hr.; if it be assumed, as is highly probable, that the concentration of phospholipid P in the glandular tissue did not change significantly during the experiment, the same amount of phospholipid as was degraded must also have been synthesized. It is possible that the fatty acids which are liberated from the phospholipids are utilized in some way in milk-fat formation, and further studies to investigate this topic are in progress.

As already mentioned, no radioactivity was detected in the milk phospholipids of the animals which received [^{32}P]phospholipids, though labelled phospholipids were found in the milk after injection of labelled inorganic phosphate (cf. Aten & Hevesy, 1938). These findings indicate that milk phospholipids may be derived to a considerable extent from phospholipids elaborated *de novo* in the gland; they lend support to the suggestion made by Popják (1951–2) that milk fat is not a random sample of the fat within the mammary gland and that the glandular epithelium may contain 'structural' lipids undergoing independent turnover. However, if this be the case, it seems that short-chain fatty acids produced from acetate in the mammary gland (see review by Popják, 1951–2) are not esterified in the synthesis of phospholipids which are secreted into the milk, since Hilditch & Maddison (1941) found that no fatty acid of shorter chain length than myristic acid was present in the phospholipids of cow's-milk lipids.

SUMMARY

1. Two lactating rabbits were injected intravenously with inorganic [^{32}P]phosphate and four lactating rabbits were injected with [^{32}P]phospholipids contained in plasma. The specific radioactivities of the phospholipids of mammary glandular tissue (and liver) were compared with the specific radioactivities of the alkali-stable (glycerophosphate) phosphorus and inorganic phosphorus from the corresponding organs.

2. It was concluded that phospholipids are synthesized by the lactating gland and also taken up from the plasma and degraded to the extent of about 20% in 2 hr.; it is probable that the same amount was also synthesized.

3. The results are briefly discussed in relation to the composition and secretion of milk lipids.

This study was commenced when the author was granted facilities by Sir Charles Harington, F.R.S., to work for a short period at the National Institute for Medical Research, London. I am very grateful to Dr G. Popják, who initiated this work, for his help and guidance. I also wish to acknowledge the skilled assistance of Mr W. R. H. Duncan and Mr J. W. C. Davis.

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The Biochemistry of Organo-tin Compounds

DIETHYLTIN DICHLORIDE AND TRIETHYLTIN SULPHATE

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The potential value of organo-tin compounds as fungicides (Van der Kerk & Luijten, 1954) has stimulated an examination of their toxicity. The older work of White (1881) has been confirmed and extended by Stoner, Barnes & Duff (1955). Both diethyltin dichloride and triethyltin sulphate are highly toxic substances, and in discussing their mode of action the authors suggest the probable importance of a biochemical lesion. Interest in these compounds is increased by the report of many deaths in France after the oral administration of diethyltin dichloride as a treatment for boils (*Brit. med. J.* 1954).

The work described in this paper represents an attempt to define their biochemical action. Stoner *et al.* (1955) show that triethyltin sulphate and diethyltin dichloride do not produce the same clinical symptoms in rats. Furthermore, rats are protected from a lethal dose of diethyltin dichloride by 2:3-dimercaptopropanol (BAL); this is not possible with triethyltin sulphate. Triethyltin compounds are at least 100 times more effective as fungicides than are diethyltin compounds (Van der Kerk & Luijten, 1954). Differences are also apparent in their action on the biochemical behaviour of rat-brain brei and rat-liver mitochondria.

Triethyltin sulphate and diethyltin dichloride ionize in aqueous solution to give the univalent triethyltin and the bivalent diethyltin ions (Luijten & Van der Kerk, 1952; Smyth, 1941). Irrespective of the extent of their ionization, we have compared the bivalent diethylstannyl and phenylarsino groups and the univalent triethylstannyl and diphenylarsino groups. Like phenylarsenious acid, diethyltin dichloride inhibits α-keto acid oxidases, a result which is in agreement with the 'dithiol theory' of Peters (1948). Triethyltin sulphate, on the other hand, has little affinity for SH groups and is a powerful inhibitor of the phosphorylation processes which are linked to oxidation.

EXPERIMENTAL

Rat-brain brei. Rats were killed by a blow on the back of the neck and the brain was quickly removed and rinsed in ice-cold 0·9% (w/v) NaCl. The tissue (1 brain is approximately 1·2 g.) was squeezed through the nozzle of a 5 ml. all-glass syringe into 6 ml. ice-cold 0·1 M sodium phosphate buffer pH 7·4. The suspension was gently sucked in and out of the syringe until a smooth dispersion was produced (6–8 times up and down is usually required). A portion (1 ml.) of this suspension was added to the substrate in phosphate buffer (total volume in flasks, 3 ml.). The centre well of each flask contained 0·15 ml. 20% (w/v) KOH for CO_2 absorption and the gas phase was air. After warming to 37°, readings of O_2 uptake were taken at intervals up to 70 min. Such preparations will continue to oxidize lactate, glucose and pyruvate for up to 3 hr.

Rat-liver mitochondria. A few of the initial experiments were carried out with rat-liver mitochondria isolated by the procedure of Schneider (1948). A modified procedure using

lower centrifugal forces and a final washing with 0·9% (w/v) KCl was used for most of the experiments. Rats were killed by a blow on the head, the liver was quickly removed and excess of blood rinsed off with distilled water. The liver, after weighing, was disrupted in 75 ml. ice-cold 0·25M sucrose, using a Nelco homogenizer (Measuring and Scientific Instruments, London) at 5000 rev./min. for 1·5 min. The 'nuclear fraction' was centrifuged at 600 *g* at 0° for 10 min. The supernatant was poured off through four layers of surgical gauze and the mitochondria were sedimented at 5000 *g* at 0° for 15 min. (Strittmatter & Ball, 1954). The supernatant was discarded and the particles were suspended in ice-cold 0·9% (w/v) KCl solution and sedimented at 2500 *g* at 0° for 5 min. After removal of the supernatant, the mitochondria were suspended in ice-cold 0·9% (w/v) KCl to a volume in ml. equal to twice the weight of the liver in g. Such preparations were stored in an ice bath until used, usually within 1 hr. of preparation. Protein determinations by the biuret method of Robinson & Hogden (1940) indicated a consistent recovery of mitochondria. Such preparations are consistently active, oxidizing all the tricarboxylic acid cycle substrates, octanoate and β-hydroxybutyrate, and esterifying inorganic phosphorus. The general technique, essentially that of Judah (1951), was as follows: Each flask contained 3 ml. of a solution containing 0·00123M adenosine 5′-phosphate (AMP), 0·0067M-$MgSO_4$, 0·045M-KCl, $7{\cdot}25 \times 10^{-6}$M cytochrome *c*, 0·0167M sodium phosphate buffer pH 7·4, 0·01M substrates as neutralized sodium or potassium salts and mitochondria equivalent to 167–333 mg. wet wt. of original liver. The centre well of each flask contained 0·15 ml. 20% (w/v) KOH for CO_2 absorption and the gas phase was air. The mitochondria in ice-cold 0·9% (w/v) KCl were added to the above components at room temp., the flasks warmed to 37° and readings taken from 10 to 70 min. Variations in this general procedure are given in the legends of the particular experiments.

Special chemicals. Succinic acid, sodium citrate, lithium lactate, glucose, *p*-phenylenediamine (British Drug Houses, Poole), adenosine 5′-phosphoric acid (AMP), sodium pyruvate and glutathione (GSH) (Roche Products Ltd., Welwyn), α-oxoglutaric acid, L-malic acid, oxaloacetic acid, sodium DL-β-hydroxybutyrate (Light and Co., Colnbrook), L-glutamic acid (Ashe Laboratories, London) and 2:3-dimercaptopropanol (BAL) (Boots Pure Drug Co., Nottingham) were used.

Cytochrome *c* was prepared from 27 lb. cow heart by the method of Keilin & Hartree (1952). The product (1·58 g.) was 73% pure, using ϵ $2{\cdot}81 \times 10^4$ for reduced cytochrome *c* at 550 mμ. and a mol. wt. of 16 500 (Umbreit, Burris & Stauffer, 1951).

Coenzyme I (Co I) was prepared from baker's yeast by the method of Kornberg & Pricer (1953). By processing 112 lb. yeast (British Fermentation Products, London) and proceeding to the acetone powder stage after removal of silver, a yield of 5·9 g. was obtained. Assay of this material by the method of Gutcho & Stewart (1948), but using ϵ $6{\cdot}22 \times 10^3$ for reduced Co I at 340 mμ. (Horecker & Kornberg, 1948) indicated a purity of 74%. A solution of reduced Co I suitable for the determination of Co I–cytochrome *c* reductase was prepared by adding 3 ml. 0·4% (w/v) $Na_2S_2O_4$ in 1% (w/v) $NaHCO_3$ to 23·2 mg. Co I. This solution was heated in a boiling-water bath for exactly 1 min., cooled in ice and diluted to 25 ml. with a cold solution containing 1% (w/v) Na_2CO_3 and 1% (w/v) $NaHCO_3$. This solution was aerated rapidly until a constant optical density at 340 mμ. was obtained. The solution was stored at +2° and before use was brought to pH 7·6 with 0·5M-KH_2PO_4.

The barium salt of adenosine triphosphoric acid (ATP) was prepared from rabbit muscle by the method of LePage (1949). The Ba salt was converted into the K salt by treatment with Amberlite 1R-120 (K-cycle).

Hexokinase was prepared from baker's yeast by the method of Berger, Slein, Colowick & Cori (1946), and the preparation carried to their stage 3*a*. When assayed by their procedure (but at 37° instead of 30°) our preparation had an activity of 5350 units/ml. When oxidative phosphorylation was being measured 100 units/flask were added.

Inhibitors. Diethyltin dichloride was used as a stock solution, 2·0 mg./ml. (8×10^{-3}M). Triethyltin hydroxide, 26·2 g. (0·118 mole), was dissolved in 130 ml. acetone, filtered and 12·0 ml. (0·060 mole) 5M-H_2SO_4 were added with vigorous stirring. A further 130 ml. acetone were added, the mixture was cooled and the solid filtered off. After washing with acetone, it was resuspended in acetone, filtered off and dried in air and finally *in vacuo*. Yield, 23·5 g. triethyltin sulphate (stock solution 10 mg./ml., $3{\cdot}94 \times 10^{-2}$M). A stock solution of phenylarsenious acid (Na salt) was prepared by adding 5 ml. 0·1N-NaOH to 30 mg. phenylarsine oxide and gently warming in a water bath at 60–70°. After addition of 5 ml. water the solution was stored at +2°. A fresh solution was prepared every week. A stock solution of diphenylchloroarsine (2·64 mg./ml.) was prepared in 60% (v/v) ethanol. Before use it was diluted 50 times with water to give a concentration of 2×10^{-4}M in 1·2% (v/v) ethanol. Further dilutions were made in 1·2% (v/v) ethanol and a maximum of 1 ml. of this solution in a total of 3 ml. was used for any experiment. *p*-Chloromercuribenzoic acid was prepared by the method of Whitmore & Woodward (1947) and a stock solution (1×10^{-3}M) was prepared by dissolving in dilute NaOH and quickly neutralizing to pH 7·4 with HCl.

Anaerobic glycolysis. Lactic acid was estimated by the technique of Barker & Summerson (1941). The same results were obtained when additional copper–lime treatments to remove interfering substances were used (Hullin & Noble, 1953). Anaerobic glycolysis was studied by using a ground rat-brain preparation, the procedure being essentially that of Krimsky & Racker (1949), see Table 4.

Reactivity of inhibitors with SH groups. The use of the nitroprusside method of Grunert & Phillips (1951) for the determination of SH groups remaining after treatment with inhibitor was unsatisfactory. The method of Stocken & Thompson (1946*b*) was modified by using 2:6-dichlorophenolindophenol in place of porphyrindin. It is well known that SH compounds interfere in the determination of L-ascorbic acid using 2:6-dichlorophenolindophenol (O'Brien, 1944), and we have found that this dye is readily reduced by GSH, cysteine, thiomalic acid, kerateine and BAL at pH 7·4 in phosphate buffer. The rate of reduction of the dye by an SH compound with and without the inhibitor is followed by reading the optical density at 620 mμ., using a Unicam D.G. spectrophotometer, see Table 1.

α-Keto acid determinations. Pyruvate was determined in rat-brain brei by the method of Friedmann & Haugen (1943), using toluene to extract the 2:4-dinitrophenylhydrazones. For the determination of the total α-keto acid produced by rat-liver mitochondria, the hydrazones were extracted by shaking with 3 ml. diethyl ether instead of

toluene. In a few experiments (cf. Table 9) the sensitivity of the method was increased fourfold by using 5 ml. trichloroacetic acid (TCA) filtrate instead of 3 ml. and extracting the hydrazones from the ether extract (3 ml.) with 2·5 ml. 10% (w/v) Na_2CO_3 solution instead of 6 ml. A portion (2 ml.) of this extract was treated with 2 ml. 1·5N-NaOH solution and the colour read after 10 min. at 520 mμ. in 1 cm. cells, using a Unicam S.P. 600 spectrophotometer. The quotient of the optical density at 420 mμ. over that at 520 mμ. was routinely determined and has been found to be $1·30 \pm 0·004$ for pyruvate and $2·04 \pm 0·011$ for α-oxoglutarate. In representative experiments we have checked the identity of the α-keto acid measured, using the chromatographic method of El Hawary & Thompson (1953). Samples from Warburg flasks were deproteinized (final concentration 9% w/v TCA) and were stored in an ice bath before the determination of α-keto acids. No precautions other than cooling have been made to prevent the breakdown of oxaloacetate to pyruvate, and chromatographic examination has shown that both are present. The total α-keto acids produced 3, 6, 9 and 12 min. after the addition of inhibitor by rat-liver mitochondria metabolizing citrate, L-malate and L-glutamate were determined. With citrate as substrate and phenylarsenious acid as inhibitor the α-keto acid level increased until the O_2 uptake had decreased by 50%. Further decreases in O_2 uptake are accompanied by a fall in α-keto acid level. Therefore, for the experiments described in Tables 6 and 7, amounts of inhibitor to produce not more than 30% inhibition of O_2 uptake were used.

Co I–cytochrome c reductase. The method of Hogeboom (1949) was used with mitochondria prepared by the method of Schneider (1948). The solution for each determination contained potassium phosphate pH 7·6 0·025M, nicotinamide 0·0205M, KCN $7·5 \times 10^{-5}$M, cytochrome *c* $1·9 \times 10^{-5}$M, reduced Co I $4·5 \times 10^{-5}$M, 0·2 ml. mitochondria suspension in 0·25M sucrose equivalent to 0·66 mg. wet wt. of original liver tissue, and water to 4 ml. The control contained no mitochondrial suspension. Triethyltin sulphate was added from a stock solution (10 mg./ml.) neutralized to pH 7·6. The rate of reduction of cytochrome *c* was followed by the rise in optical density at 549 mμ., using 1 cm. cells in a Unicam S.P. 650 quartz spectrophotometer. The rate of reduction was calculated from the early straight line portion of the curve. No reduction of cytochrome *c* occurred without the addition of reduced Co I or mitochondrial suspension. All determinations were at room temp. (18–20°).

Dehydrogenases. A technique was used in which high concentrations of keto acid blocking agents have been

Table 1. *Reaction of organo-tin, organo-arsenic and organo-mercury compounds with* SH *groups of glutathione and* 2:3-*dimercaptopropanol*

The solution of SH compound (1·0 ml.) was added to a mixture of 1·0 ml. solution of the compounds to be tested and 7 ml. 0·067M phosphate buffer pH 7·4. After 5 min. 1·0 ml. 2:6-dichlorophenolindophenol (70–90 μg.) was added and the rate of decolorization determined from the decrease in optical density at 620 mμ., using a Unicam D.G. spectrophotometer. Room temperature 18–20°. Under these conditions there is a linear relation between dye concentration and optical density. The time for 40% decolorization was determined graphically. *R* is the quotient of the molar concentration of organo-metal ion over the molar concentration of SH compound.

Concn. of SH compound (M)	Concn. of organo-metal compound (M)	*R*	Time for 40% decolorization: No organo-metal compound (sec.)	Time for 40% decolorization: With organo-metal compound (sec.)
	p-Chloromercuribenzoic acid			
GSH, 6×10^{-5}	$8·1 \times 10^{-5}$	1·3	118	∞
BAL, $2·3 \times 10^{-5}$	$8·1 \times 10^{-5}$	3·2	38	∞
	Phenylarsenious acid			
GSH, $5·1 \times 10^{-5}$	$8·9 \times 10^{-4}$	17·4	125	750
BAL, $2·2 \times 10^{-5}$	$4·4 \times 10^{-5}$	2·0	46	10% in 15 min.
	Diphenylchloroarsine			
GSH, $5·1 \times 10^{-5}$	$2·0 \times 10^{-4}$	3·9	125	10% in 30 min.
BAL, $2·2 \times 10^{-5}$	$1·0 \times 10^{-4}$	4·6	46	∞*
	Arsenious acid			
GSH, 5×10^{-5}	$1·8 \times 10^{-3}$	36	118	118
BAL, $2·3 \times 10^{-5}$	$1·8 \times 10^{-3}$	78	38	∞
	Triethyltin sulphate			
GSH, 5×10^{-5}	$7·8 \times 10^{-4}$	15·6	118	135
BAL, $2·3 \times 10^{-5}$	$7·8 \times 10^{-4}$	33·9	38	55
	Diethyltin dichloride			
GSH, 5×10^{-5}	$9·7 \times 10^{-4}$	19·4	112	172
BAL, $2·3 \times 10^{-5}$	$9·7 \times 10^{-4}$	42·2	38	∞
BAL, $2·2 \times 10^{-5}$	$4·5 \times 10^{-5}$	2·0	46	6% in 15 min.

* The solution became slightly turbid, but visually there was no appreciable decolorization of the dye.

avoided. The conditions necessary, i.e. Co I concentration, pH and substrate concentration, to give a reasonable amount of Co I reduction have been calculated from the equilibrium constants of lactic dehydrogenase (Racker, 1950), L-malic dehydrogenase (Burton & Wilson, 1953) and L-glutamic dehydrogenase (Olson & Anfinsen, 1953). The rate of reduction of Co I was followed by the rise in optical density at 340 mμ. in 1 cm. quartz cells, using a Unicam S.P. 650 spectrophotometer. The details of the experiments with an extract of acetone-dried rat liver and rat-liver mitochondria are given in Table 10. In order to use a low Co I concentration lactic dehydrogenase was studied by catalysing the reduction of pyruvic acid to lactic acid by reduced Co I. The equilibrium point of this reaction at pH 7·4 favours the reaction proceeding in this direction (Racker, 1950). The solution for each determination contained potassium phosphate pH 7·6 0·025M, nicotinamide 0·0205M, KCN $7{\cdot}5 \times 10^{-5}$M, sodium pyruvate 0·01M, reduced Co I $1{\cdot}04 \times 10^{-4}$M, 0·4 ml. of a rat-liver extract (cf. Table 10) equivalent to 0·8 mg. original acetone-dried rat liver, and water to 4 ml. The control contained no Co I. Triethyltin sulphate was added from a stock solution (10 mg./ml.) neutralized to pH 7·6. The rate of oxidation of reduced Co I was followed by the fall in optical density at 340 mμ. in 1 cm. quartz cells and was calculated from the linear portion of the curve. The rate of oxidation of reduced Co I was negligible without pyruvate. All determinations were made at room temp.

Oxidative phosphorylation. The technique involved the conversion of ATP formed into glucose 6-phosphate by hexokinase. The details of the medium used are given in Table 13. Inorganic phosphorus was determined by the method of Fiske & Subbarow (1925) after deproteinization with a final concentration of 3% (w/v) TCA. The TCA (0·5 ml. 60%, w/v) was tipped from the side arm on the contents of the flasks at various times, followed later by 6·5 ml. water.

RESULTS

Affinity for SH groups of glutathione and BAL

Mercurials and tervalent arsenicals interact with enzymes requiring SH groups (Hellerman, 1937; Barron & Singer, 1945; Peters, Sinclair & Thompson, 1946; Stocken & Thompson, 1946*a*). It was natural to suspect that compounds of tin would interfere with enzymes in a similar way.

Diethyltin dichloride. The reduction of the oxidation–reduction indicator 2:6-dichlorophenolindophenol by both GSH and BAL is completely prevented by *p*-chloromercuribenzoic acid at molecular quotients as low as 1·3 per molecule of SH compound (Table 1). Similarly, dye reduction by BAL is prevented by phenylarsenious acid and arsenious acid, whereas their affinity for the SH groups of glutathione is much less. Diethyltin dichloride reacts avidly with BAL, but only slightly with GSH. Thus the affinity of diethyltin dichloride for SH groups closely resembles that of phenylarsenious acid, arsenious acid and lewisite (Stocken & Thompson, 1946*a*).

Triethyltin sulphate. Triethyltin sulphate has little affinity for either GSH or BAL (Table 1). In this respect it is unlike *p*-chloromercuribenzoic acid and diphenylchloroarsine, which have marked affinity for monothiols (Barron & Singer, 1945; Lotspeich & Peters, 1951).

Experiments with rat-brain brei

Diethyltin dichloride. Diethyltin dichloride (10^{-4}–10^{-5}M) prevents oxygen uptake of rat-brain brei when glucose, lactate and pyruvate are used as substrates. Determinations of pyruvate levels have been made in brain brei metabolizing lactate in the presence of arsenious acid, phenylarsenious acid and diethyltin dichloride. Diethyltin dichloride leads to an accumulation of pyruvate when the oxygen uptake is lowered and in this respect resembles arsenious acid and phenylarsenious acid (Table 2). Experiments have shown that with all

Table 2. *Pyruvate levels in rat-brain brei oxidizing lactate in the presence of various inhibitors*

Each flask contained lithium lactate 0·015M, brei equivalent to 17 mg. wet wt. of original brain and inhibitor as indicated. The final volume was made up to 3·0 ml. with 0·1M phosphate buffer pH 7·4. The centre well contained 0·15 ml. 20% (w/v) KOH for CO_2 absorption, except for the experiments with cyanide, when mixtures of KOH and KCN were used (Krebs, 1935). The gas phase was air and the temperature 37°. After 10 min. equilibration readings were taken at 10, 40 and 70 min. At the end of the experiment 3·0 ml. 18% (w/v) TCA were added to each flask and 3·0 ml. samples taken for the estimation of pyruvate. Values from control flasks without substrate, with and without inhibitor, were subtracted from the corresponding values for flasks containing lactate. The difference between the controls with and without lactate was approximately 23 μg. pyruvate/flask.

Concn. of inhibitor (M)	O_2 uptake (% of control)	Pyruvate level (% of control)
Arsenious acid		
$7{\cdot}1 \times 10^{-6}$	85	104
$2{\cdot}1 \times 10^{-5}$	76	159
$5{\cdot}0 \times 10^{-5}$	59	282
Phenylarsenious acid		
$9{\cdot}9 \times 10^{-6}$	65	140
$3{\cdot}0 \times 10^{-5}$	13	490
Triethyltin sulphate		
$8{\cdot}7 \times 10^{-6}$	89	82
$1{\cdot}9 \times 10^{-5}$	76	—
$4{\cdot}4 \times 10^{-5}$	46	48
$2{\cdot}0 \times 10^{-4}$	19	24
Diethyltin dichloride		
$1{\cdot}4 \times 10^{-4}$	74	131
$2{\cdot}7 \times 10^{-4}$	44	263
$8{\cdot}1 \times 10^{-4}$	34	443
Cyanide		
$3{\cdot}0 \times 10^{-6}$	95	93
$3{\cdot}0 \times 10^{-5}$	43	31

Table 3. *Effect of* SH *compounds upon the inhibition of pyruvate oxidation of rat-brain brei by various inhibitors*

Each flask contained 0·01 M pyruvate. SH compounds and inhibitors were added as indicated and left 5 min. at room temp. before 1·0 ml. of brain brei (equivalent to 170 mg. wet weight of original brain) was added. The final volume was made up to 3·0 ml. with 0·1 M phosphate buffer pH 7·4. After warming up for 10 min. to 37° readings were taken at 10, 40 and 70 min. In each case a control flask without substrate was run and the value subtracted from the corresponding flask containing substrate. *R* is the quotient of the molar concentration of SH compound/molar concentration of inhibitor.

Concn. of SH compound (M)	Concn. of inhibitor (M)	*R*	O_2 uptake (% of control)	
			Inhibitor	Inhibitor + SH compound
	Arsenious acid			
GSH, 5×10^{-3}	6×10^{-5}	84	18	20
BAL, $3{\cdot}8 \times 10^{-4}$	6×10^{-5}	6·3	13	73
	Diethyltin dichloride			
GSH, 5×10^{-3}	$1{\cdot}7 \times 10^{-4}$	29	36	68
BAL, $3{\cdot}8 \times 10^{-4}$	$1{\cdot}7 \times 10^{-4}$	2	45	87
	Triethyltin sulphate			
GSH, 5×10^{-3}	$8{\cdot}2 \times 10^{-5}$	61	18	27
BAL, $3{\cdot}8 \times 10^{-4}$	$6{\cdot}5 \times 10^{-5}$	6	25	17

three inhibitors this accumulation is prevented by BAL. Rat-brain brei is also protected by BAL from the inhibition of pyruvate oxidation by arsenious acid and diethyltin dichloride (Table 3). GSH, in higher concentration, is ineffective against arsenious acid inhibition and only partially effective against diethyltin dichloride inhibition.

Triethyltin sulphate. Triethyltin sulphate (10^{-4}–10^{-5} M) lowers oxygen uptake of rat-brain brei with glucose, lactate and pyruvate as substrates. In contrast to diethyltin dichloride with lactate as substrate, triethyltin sulphate lowers both oxygen uptake and pyruvate concentration (Table 2). Neither BAL nor GSH influences the lowered level of pyruvate or the inhibition of oxygen uptake with pyruvate as substrate (Table 3). A lowering of pyruvate level could theoretically be due to either an increased utilization or to a decreased production of pyruvate. The former is improbable with a lowered oxygen consumption. An inhibition of lactic dehydrogenase would produce less pyruvate, and anaerobic glycolysis would also be prevented. Little or no inhibition of anaerobic glycolysis is obtained at concentrations which will inhibit lactate oxidation in brain brei (Table 4). At concentrations over 10^{-3} M complete inhibition is obtained. No explanation of this inhibition can be given, but it should be noted that diethyltin dichloride also inhibits at these concentrations and phenylarsenious acid produces considerable inhibition at 4×10^{-5} M. It is clear that the lowering of pyruvate concentration cannot be due to a specific inhibition of lactic dehydrogenase, and this is confirmed by spectrophotometric measurements of the rate of reduction of Co I by a preparation of lactic dehydrogenase from rat liver (cf. Table 10).

Table 4. *Effect of triethyltin sulphate upon anaerobic glycolysis by rat-brain dispersions*

A rat brain (approx. 1·2 g.) was placed in a mortar surrounded by an ice and salt freezing mixture and ground until it had frozen to the sides of the mortar. Water (4·8 ml.) was added, forming a thick paste, followed by 6·0 ml. 0·02 M phosphate buffer pH 7·4 to give a 10% dispersion of brain, 0·3 ml. of which was added to a solution of the following substances, giving the final concentrations of $KHCO_3$ 0·016 M, $(NH_4)_2HPO_4$ 0·02 M, $MgCl_2$ 0·007 M, nicotinamide 0·033 M, ATP 0·001 M, Co I $9{\cdot}45 \times 10^{-4}$ M, glucose 0·024 M. The tubes were incubated for 30 min. at 37°, 5·8 ml. water and 1·2 ml. 60% (w/v) TCA added. After centrifuging, lactic acid was determined in the supernatant. No difference in lactate production, using anaerobic or aerobic conditions, was found.

Concn. of triethyltin sulphate (M)	Lactic acid produced (mg./100 mg. wet wt. brain/30 min.)	
	Expt. 1	Expt. 2
Nil	4·90	5·25
$1{\cdot}39 \times 10^{-6}$	4·97	5·25
$6{\cdot}96 \times 10^{-6}$	4·67	5·11
$3{\cdot}48 \times 10^{-5}$	4·42	4·98
$1{\cdot}74 \times 10^{-4}$	4·50	4·90
$8{\cdot}72 \times 10^{-4}$	3·62	4·42
$4{\cdot}36 \times 10^{-3}$	Nil	0·28

Since lactic dehydrogenase is not inhibited by triethyltin sulphate, other components of the respiratory chain were considered. Inhibition of cytochrome oxidase by cyanide produces a lowering of pyruvate levels (Table 2). Triethyltin sulphate, unlike cyanide, does not inhibit succinate oxidation at the concentrations which inhibit the oxidation of lactate and pyruvate. At higher concentrations (2×10^{-4} M) the effect varies with the concentration

of substrate. With 0·06 M succinate there is an inhibition of oxidation, while with 0·009 M substrate there is invariably a stimulation. No detailed explanation can at present be given of these findings.

Experiments with rat-liver mitochondria

Diethyltin dichloride. The effect of inhibitors on the oxidation of various substrates by rat-liver mitochondria (Table 5) are given as the working range of inhibitor concentrations because it is difficult to obtain a precise amount of inhibition from experiment to experiment. With the exception of succinate, all the substrates of the tricarboxylic acid cycle are strongly inhibited by phenylarsenious acid and diethyltin dichloride. results are obtained with all three substrates (Table 6) estimating α-keto acids during the early part of the experiments (3–12 min.). For these experiments the inhibitors were tipped in from the side arm to the actively metabolizing mitochondria. More inhibitor is required to produce a given amount of inhibition by this procedure than when fresh mitochondria are added directly to the inhibitor and substrate. It has been our experience that α-keto acid levels are a better early index of enzyme inactivation than is oxygen uptake, and we have used this technique to examine the protective effect of SH compounds. The results in Table 7 make it clear that BAL prevents the α-keto acid accumulation produced by both diethyltin dichloride and phenylarsenious acid, whereas GSH is ineffective.

Table 5. *The inhibitory range of various substances upon the oxidation of substrates by rat-liver mitochondria*

Each flask contained 3 ml. of a solution of AMP 0·00123 M, $MgSO_4$ 0·0067 M, KCl 0·045 M, sodium phosphate pH 7·4 0·0167 M, cytochrome *c* 7·3 × 10^{-6} M, substrate 0·01 M (β-hydroxybutyrate 0·1 M, *p*-phenylenediamine 0·03 M), mitochondria (0·5 ml.) in 0·9% (w/v) KCl and various inhibitors. Mitochondria were added to the above components at room temperature, warmed to 37° for 10 min. and readings taken for 10–70 min. pI = − log (molar concentration of inhibitor). Where it was not possible to produce complete inhibition, the inhibition obtained with the concentration of inhibitor stated is given in brackets.

	Inhibitors			
	Phenylarsenious acid	Diethyltin dichloride	Diphenylchloroarsine	Triethyltin sulphate
Substrate	pI			
L-Malate	6·0–6·5	4·5–5·5	4·5–5·4	4·7–5·7
Citrate	5·3–6·0	3·8–4·8	5·0–5·7	4·5–5·5
α-Oxoglutarate	5·7–6·5	4·3–5·3	4·7–5·4	4·2–5·2
Succinate	4·5 (25%)	3·9 (25%)	4·2 (25%)	3·9 (40%)
Pyruvate	6·0–7·0	4·5–5·5	4·4–5·4	4·2–5·2
L-Glutamate	6·0–6·5	4·3–5·3	4·4–5·1	4·5–5·5
p-Phenylenediamine	3·5 (20%)	3·9 (20%)	—	3·9 (0%)
β-Hydroxybutrate	4·4–5·0	—	—	4·5–5·5

The oxidation of L-glutamate is also inhibited, but the oxidation of succinate is only slightly inhibited. With ferricyanide as an electron acceptor instead of oxygen, diethyltin dichloride and phenylarsenious acid prevent the oxidation of L-malate, citrate and α-oxoglutarate. The concentrations of diethyltin dichloride necessary for inhibition under these conditions are less than when measuring oxygen uptake, whereas phenylarsenious acid is equally effective for both procedures. The oxidation of succinate was much less affected by both diethyltin dichloride and phenylarsenious acid than the oxidation of the other three substrates.

An examination has been made of the influence of these inhibitors on the α-keto acid concentrations when citrate, L-glutamate and L-malate are used as substrates. With citrate, both diethyltin dichloride and phenylarsenious acid produce a continuous rise in α-keto acid concentration (Fig. 1). Similar

Triethyltin sulphate. Triethyltin sulphate and diphenylchloroarsine inhibit the oxidation of L-glutamate and all the tricarboxylic acid cycle substrates except succinate (Table 5). Succinate oxidation is only slightly inhibited by higher concentrations of both inhibitors. Triethyltin sulphate inhibits β-hydroxybutyrate oxidation at the same concentration as it inhibits the substrates of the tricarboxylic acid cycle. With citrate as a substrate there is a primary depression of α-keto acid levels, followed later (30–70 min.) by a slight accumulation (Fig. 1). The results in Table 6 show that triethyltin sulphate consistently produces an early depression of α-keto acid levels when either L-malate, L-glutamate or citrate is used as substrate. Diphenylchloroarsine produces some accumulation with L-malate and citrate, but has no effect on the α-keto acid levels with L-glutamate as substrate. Neither BAL nor GSH influences the fall in α-keto acid level

produced by triethyltin sulphate with citrate as substrate (Table 7).

It is clear that triethyltin sulphate is a potent inhibitor of oxidation of tricarboxylic acid cycle substrates (except succinate) by rat-liver mitochondria. With lactate oxidation by rat-brain brei it produces a lowering of pyruvate concentration, and similarly with the oxidation of L-malate, L-glutamate and citrate it produces a primary lowering of α-keto acid levels. Such symptoms could be produced by a block at some stage in the respiratory chain. Triethyltin sulphate does not inhibit cytochrome oxidase, determined by using *p*-phenylenediamine as substrate (Table 5), and this is confirmed by the measurements of reduced cytochrome *c* in the flasks at the end of a manometric experiment using L-glutamate as substrate (Table 8). More cytochrome *c* was always found in the oxidized state than in the control, and triethyltin sulphate lowered the amount of reduced cytochrome *c* found in the presence of cyanide. Similar results have been obtained with L-malate as substrate. The results given in Table 9 show that the inhibition of succinate oxidation (Table 5) was due to depletion of substrate. In the presence of triethyltin sulphate 1 atom of oxygen is consumed for every mol. of succinate, whereas in the control 7 atoms of oxygen are used. For the experiments described in Table 5, 30 μmol. of succinate per flask were used. This amount of succinate would have been completely oxidized in the presence of triethyltin sulphate, whereas in the controls only a small proportion would have been removed. In our experiments using ferricyanide as electron acceptor we found no inhibition of succinate oxidation. This is explained by the short time of the experiment (35 min.) and the use of less mitochondria, because there is an evolution of 2 moles of CO_2 for every atom of oxygen. It is concluded from these experiments that succinic oxidase is not inhibited by triethyltin sulphate. Therefore, on the basis of current views (Keilin & Slater, 1953), the respiratory chain from oxygen down as far as the succinic-cytochrome *c* factor (Slater, 1949; Clark, Neufeld, Widmer & Stotz, 1954) is functional in the presence of triethyltin sulphate. The remaining components down to and including the dehydrogenases have been examined. Co I–cytochrome *c* reductase has been determined by the rate of reduction of cytochrome *c* in the presence of reduced Co I. This rate is not altered by 5×10^{-4}M triethyltin sulphate. The

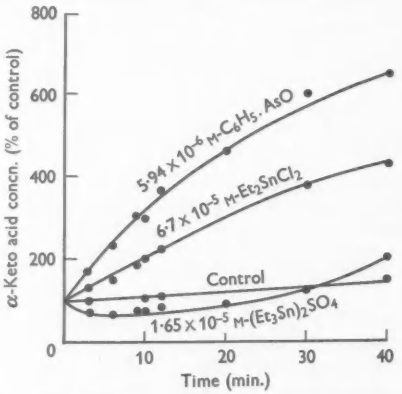

Fig. 1. α-Keto acid concentration at various times after treatment of rat-liver mitochondria oxidizing citrate with various inhibitors. The components of the flask are as given in Table 5. Mitochondria equivalent to 167 mg. wet wt. of original liver were added at room temp., warmed to 37° for 10 min. and the inhibitor or water tipped in from the side arm. At various times 3 ml. 18% (w/v) TCA were added and the total α-keto acids determined. The inhibition of O_2 uptake for all three inhibitors was 23–27%. The ratios of optical densities at 420 and 520 mμ. indicated that 68–75% of the keto acids were α-oxoglutarate.

Table 6. *α-Keto acid levels produced by rat-liver mitochondria with citrate, L-malate and L-glutamate as substrates in the presence of various inhibitors*

The components of the flasks were as given in Table 5. Mitochondria (equivalent to 167 mg. wet wt. of original liver) were added to the flasks at room temp. They were warmed to 37° for 10 min. and the inhibitor or water was tipped in from the side arm. After the times indicated 3 ml. 18% (w/v) TCA were added and total α-keto acids determined.

Substrate	Time with inhibitor (min.) 3	6	9	12	Control after 12 min.
	α-Keto acid concentration (% of control at 3 min.)				
Phenylarsenious acid, $5{\cdot}9 \times 10^{-6}$M					
L-Malate	176	240	310	380	—
L-Glutamate	240	303	386	396	122
Citrate	160	204	253	288	120
Diethyltin dichloride, $6{\cdot}7 \times 10^{-5}$M					
L-Malate	124	134	159	185	89
L-Glutamate	200	277	295	317	115
Citrate	144	159	202	248	121
Diphenylchloroarsine, $6{\cdot}7 \times 10^{-5}$M					
L-Malate	134	164	150	187	97
L-Glutamate	125	120	107	105	118
Citrate	123	134	—	158	127
Triethyltin sulphate, $1{\cdot}6 \times 10^{-5}$M					
L-Malate	62	62	59	70	104
L-Glutamate	80	—	88	99	121
Citrate	71	67	75	85	101

activity of the lactic, L-malic or L-glutamic dehydrogenases of rat-liver extract or the L-malic or L-glutamic dehydrogenases of rat-liver mitochondria is not affected by 4×10^{-4}M triethyltin sulphate (Table 10). In agreement with the results of Christie & Judah (1953) we find that treatment of mitochondria with water greatly increases the rate of Co I reduction with L-malate or L-glutamate as substrate. This increased activity is not inhibited by triethyltin sulphate.

Table 7. *Prevention by SH compounds of the accumulation of α-keto acids by rat-liver mitochondria oxidizing citrate in the presence of various inhibitors*

Each flask contained 3 ml. of a solution of AMP 0·00123 M, $MgSO_4$ 0·0067 M, KCl 0·065 M, sodium phosphate pH 7·4 0·0167 M, cytochrome *c* $7{\cdot}3 \times 10^{-6}$ M, sodium citrate 0·01 M, mitochondria equivalent to 333 mg. wet wt. of original liver. Mitochondria were added at room temp. to the solution of these components together with the SH compound, warmed to 37° for 2 min., and the inhibitor or water was tipped in from the side arm. After 6 min. at 37° 3 ml. 18% (w/v) TCA were added and the total α-keto acids determined. The controls contained approximately 65 μg. α-oxoglutarate. The quotient of the optical densities 420 mμ./520 mμ. was 2·06 ± 0·013. *R* is the quotient of the molar concentration of SH compound/molar concentration of the inhibitor.

Concn. of SH compound (M)	*R*	α-Keto acid concentration (% of control): SH compound	Inhibitor	SH compound + inhibitor
Phenylarsenious acid, $5{\cdot}9 \times 10^{-6}$ M				
GSH, $1{\cdot}23 \times 10^{-4}$	20·8	102	260	310
BAL, $6{\cdot}9 \times 10^{-5}$	11·7	88	294	89
Diethyltin dichloride, $7{\cdot}8 \times 10^{-5}$ M				
GSH, $1{\cdot}61 \times 10^{-3}$	20·7	66	202	152
BAL, $8{\cdot}6 \times 10^{-4}$	11·0	113	157	113
Triethyltin sulphate, $3{\cdot}3 \times 10^{-5}$ M				
GSH, $6{\cdot}6 \times 10^{-4}$	20·0	86	54	53
BAL, $3{\cdot}3 \times 10^{-4}$	10·0	116	56	59

Since both the triethylstannyl group and oxidized Co I are positively-charged ions the possibility of competition was considered. The equilibrium point of the reaction lactate⇌pyruvate at pH 7·4 is very much in favour of lactate formation and therefore minimal concentrations of Co I may be used (1×10^{-4} M). Triethyltin sulphate at 100 times this concentration has no effect upon the rate of oxidation of reduced Co I by pyruvate in the presence of rat-liver extract. It appears therefore that the respiratory chain is unaffected by triethyltin sulphate. This conclusion has been deduced from experiments using techniques very different from the manometric methods. However, experiments with β-hydroxybutyrate, using the mano-

Table 8. *The levels of reduced cytochrome c produced by rat-liver mitochondria oxidizing L-glutamate in the presence of triethyltin sulphate and cyanide*

The components of the flasks were as given in Table 5 except for an increased cytochrome *c* concentration, $5{\cdot}1 \times 10^{-4}$ M. For the flasks containing cyanide, CO_2 was absorbed by a mixture of 10 ml. N-KCN and 1 ml. N-KOH (Krebs, 1935). O_2 uptake was measured from 10 to 25 min. The flasks were then quickly cooled in ice and the contents centrifuged at 0° at 10 000 *g* for 10 min. The optical density of the clear supernatant was determined in 1 cm. cells at 549 mμ. The concentration of cytochrome *c* was determined by the optical density at 549 mμ. after the addition of 1 drop 10% (w/v) $K_3Fe(CN)_6$. This was done in preference to reduction with $Na_2S_2O_4$, since in the presence of triethyltin sulphate cloudiness was produced.

Additions	O_2 uptake (μl., 10–25 min.)	Reduced cytochrome *c* (% of total)
Nil	85	10
KCN, 1×10^{-3} M	8	58
Triethyltin sulphate, $9{\cdot}9 \times 10^{-4}$ M	0	5
KCN, 1×10^{-3} M + Triethyltin sulphate, $9{\cdot}9 \times 10^{-4}$ M	0	27

Table 9. *The oxidation of succinate by rat-liver mitochondria in the presence of triethyltin sulphate*

The components in the flask were as given in Table 7 with succinate in the amounts stated and triethyltin sulphate $2{\cdot}6 \times 10^{-4}$ M where indicated. Mitochondria (equivalent to 333 mg. wet wt. of original liver) were added at room temp. to a solution of all components, the flasks warmed to 37° and readings taken from 5 min. until O_2 uptake ceased. For the control experiments the O_2 uptake was extrapolated graphically to zero time and corrected for O_2 uptake obtained without substrate. O_2 uptake had almost ceased by 1 hr. For the experiments in the presence of triethyltin sulphate the O_2 uptake for the first amount of succinate was extrapolated graphically to zero time. The second amount of succinate was tipped in from the side arm when O_2 uptake had ceased (30 min.). Each value is the mean of two results.

System	Succinate added (μ-moles)	O_2 uptake (μ-atom)	μ-atom O_2 / μ-mole succinate
Control	5·62	39·6	7·05
Triethyltin sulphate, $2{\cdot}6 \times 10^{-4}$ M	22·5 (1st addition)	25·0	1·11
Triethyltin sulphate, $2{\cdot}6 \times 10^{-4}$ M	22·5 (2nd addition)	26·4	1·17
No AMP	22·5 (2nd addition)	24·5	1·08

metric technique, have shown that the same conclusion is valid.

Provided that Co I is added, β-hydroxybutyrate can be oxidized by rat-liver mitochondria, while phosphorylation (measured by the rate of incorporation of ^{32}P into esterified phosphorus) is absent (Lehninger, 1949). The oxidation of β-hydroxybutyrate is a one-step oxidation, the oxygen consumed being equivalent to the acetoacetate formed (Lehninger, 1949; Walker, 1954).

Table 10. *Effect of triethyltin sulphate upon dehydrogenases of rat-liver extract and rat-liver mitochondria*

Each solution (4 ml.) contained the following constituents; sodium phosphate 0·1 M, substrate $3·75 \times 10^{-2}$ M, KCN $2·2 \times 10^{-4}$ M, Co I $2·7 \times 10^{-3}$ M, and $3·96 \times 10^{-6}$ M triethyltin sulphate where indicated. An extract of acetone-dried rat liver was prepared by homogenizing 2 g. powder in 20 ml. 0·1 M phosphate buffer, pH 7·8. The suspension was centrifuged (5000 *g*) for 20 min. at 0°. The almost clear yellow extract was diluted with water and 0·5 ml. of a 50-fold dilution used for each determination. Rat-liver mitochondria suspended in 0·9% (w/v) KCl were centrifuged at 2500 *g* for 5 min. and suspended in either ice-cold 0·9% KCl or water to give a solution equivalent to 83 mg. wet wt. of original liver/ml. Readings have been taken at room temp. (18–20°) in 1 cm. quartz cells, using the Unicam S.P. 650 quartz spectrophotometer. Control tube contained no Co I. Blank reduction rates were determined without substrate. Rates of reduction have been calculated from the time taken for the optical density at 340 mμ. to rise from 0·35 to 0·45 equivalent to the reduction of 0·0644 μ-mole Co I/4 ml. The pH was checked at end of the experiments with a glass electrode.

Substrate	pH	Co I reduction: Control	Co I reduction: With triethyltin sulphate
		(μ-mole/100 mg. acetone powder/hr.)	
Rat-liver extract			
Lactate	7·56	500	500
L-Glutamate	7·56	140	140
L-Malate	7·81	270	270
KCl-suspended mitochondria		(μ-mole/g. original liver/hr.)	
L-Glutamate	7·56	15	24
L-Malate	7·81	13	18
Water-suspended mitochondria			
L-Glutamate	7·56	130	121
L-Malate	7·81	127	140

If Co I is present oxygen uptake is normal for 15 min. without the addition of either AMP or magnesium. Under these conditions 4×10^{-5} M triethyltin sulphate has little or no effect upon the oxidation of β-hydroxybutyrate (Table 11). The protective effect of Co I during the early part of the experiment diminishes later (Table 12, Fig. 2).

Co I also partially prevents the inhibition of L-malate oxidation by triethyltin sulphate (Table 12). This effect diminishes rapidly, and Co I is not as effective with L-malate as with β-hydroxybutyrate as substrate. The respiratory chain in the early periods of the experiment in the presence of Co I is functional and the inhibition produced later is probably due to some other process.

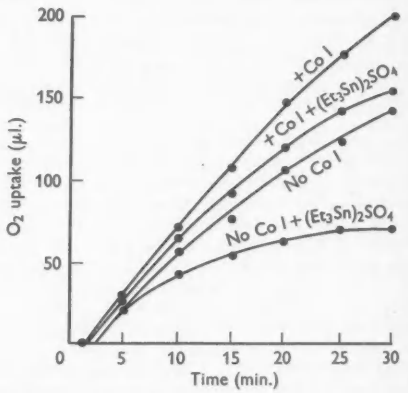

Fig. 2. The effect of Co I on the oxidation of β-hydroxybutyrate by rat-liver mitochondria with and without triethyltin sulphate. The components of the flasks were as given in Table 12. The substrate (0·1 M) and triethyltin sulphate ($2·2 \times 10^{-5}$ M) were in the side arm. Mitochondria (equivalent to 250 mg. wet wt. of original liver tissue) were added to the ice-cold contents of the flasks, warmed to 37° for 5 min., a zero reading taken and the substrate and inhibitor tipped in at 1 min.

Table 11. *Effect of AMP, magnesium, Co I and triethyltin sulphate on the oxidation of β-hydroxybutyrate by rat-liver mitochondria*

Each flask contained 3 ml. of a solution of KCl 0·045 M, sodium phosphate pH 7·4 0·0167 M, cytochrome *c* $7·3 \times 10^{-6}$ M, nicotinamide 0·02 M and mitochondria equivalent to 250 mg. wet wt. of original liver, β-hydroxybutyrate 0·1 M, Co I $3·66 \times 10^{-4}$ M and, where indicated, AMP 0·00123 M, $MgSO_4$ 0·0067 M and triethyltin sulphate $4·4 \times 10^{-5}$ M. The substrate and inhibitor were in the side arm. Mitochondria were added at room temp. to the solution of the above components and, after warming for 5 min. to 37°, the substrate and inhibitor tipped in from the side arm and readings taken every 5 min.

System	O_2 uptake (μl., 5–15 min.): Control	O_2 uptake (μl., 5–15 min.): With triethyltin sulphate
AMP, Mg, Co I	69	57
Mg, Co I	55	51
AMP, Co I	68	74
Co I	70	69

The maintenance of a low water content and a higher potassium concentration within mitochondria is a process which requires metabolism or 'high-energy' phosphates (Macfarlane & Spencer, 1953; Price & Davies, 1954; Brenner-Holzach & Raaflaub, 1954). In a medium containing magnesium sulphate, potassium chloride, sodium phosphate pH 7·4 and cytochrome *c*, but without AMP, succinate is the only acid of the tricarboxylic acid cycle oxidized. The oxidation of pyruvate, citrate, α-oxoglutarate, L-glutamate and L-malate is entirely prevented, and for the oxidation of 1 mol. succinate only 1 atom oxygen is consumed (Table 9). In this respect the effect of triethyltin sulphate resembles a system without a phosphate acceptor. Triethyltin sulphate could produce the biochemical symptoms previously described, by preventing the formation of ATP, and the results in Table 13 show that it is a potent inhibitor of the phosphorylation processes associated with the oxidation of β-hydroxybutyrate, citrate and succinate. The inhibition of inorganic phosphate uptake is always more than the inhibition of oxygen uptake. Inhibition of oxidation is therefore a secondary process. Triethyltin sulphate is as efficient an inhibitor of phosphorylation as 2:4-dinitrophenol.

Table 12. *Effect of Co I on the oxidation of β-hydroxybutyrate and* L-*malate by rat-liver mitochondria in the presence of triethyltin sulphate*

Each flask contained 3 ml. of a solution of AMP 0·00123 M, $MgSO_4$ 0·0067 M, KCl 0·045 M, sodium phosphate pH 7·4 0·0167 M, cytochrome *c* $7{\cdot}3 \times 10^{-6}$ M, nicotinamide 0·02 M and mitochondria equivalent to 167 mg. wet wt. of original liver. Where indicated, Co I $3{\cdot}66 \times 10^{-4}$ M, β-hydroxybutyrate 0·1 M and L-malate 0·01 M were added. Mitochondria were added to the solution of the above components at room temp. warmed to 37° for 10 min. and readings taken every 5 min.

Concn. of triethyltin sulphate (M)	O_2 uptake (μl.) 10–20 min.		20–30 min.	
	No Co I	With Co I	No Co I	With Co I
	β-Hydroxybutyrate oxidation			
Nil	60	61	41	45
$1{\cdot}32 \times 10^{-4}$	3	27	0	11
$4{\cdot}4 \times 10^{-5}$	3	45	0	25
$1{\cdot}47 \times 10^{-5}$	39	57	24	32
$4{\cdot}9 \times 10^{-6}$	58	62	28	42
	L-Malate oxidation			
Nil	57	71	52	68
$1{\cdot}32 \times 10^{-4}$	10	30	11	11
$4{\cdot}4 \times 10^{-5}$	10	29	2	21
$1{\cdot}47 \times 10^{-5}$	33	56	24	30

DISCUSSION

Diethyltin dichloride and triethyltin sulphate influence the biochemical mechanisms of rat-brain brei and rat-liver mitochondria in different ways. Phenylarsenious acid and diphenylchloroarsine are also dissimilar in this respect (Peters, 1952). Diethyltin dichloride and phenylarsenious acid produce a similar response, but the biochemical symptoms produced by triethyltin sulphate and diphenylchloroarsine are not identical. Previous

Table 13. *The inhibition of oxidative phosphorylation by triethyltin sulphate and 2:4-dinitrophenol*

Each flask contained 3·0 ml. of a solution of AMP 0·00123 M, $MgSO_4$ 0·0067 M, KCl 0·045 M, cytochrome *c* $7{\cdot}3 \times 10^{-6}$ M, sodium phosphate buffer pH 7·4 0·0167 M, NaF 0·008 M, sodium glycylglycine buffer pH 7·4 0·0165 M, glucose 0·0074 M, yeast hexokinase 100 units and mitochondria equivalent to 250 mg. wet wt. of original liver. Where indicated, β-hydroxybutyrate 0·1 M and succinate and citrate 0·01 M were used. The mitochondria were added to the solution of the above components at 0°, warmed to 37° for 5 min. and readings taken at 5, 10 and 15 min. Inorganic phosphorus was determined at 5 min. on a duplicate set of flasks. TCA (0·5 ml., 60%, w/v) was tipped in from the side arm for deproteinization. The results are expressed as a percentage of the control O_2 or inorganic phosphorus uptake for the 5–15 min. period. The concentrations of triethyltin sulphate used do not inhibit hexokinase.

Concn. of triethyltin sulphate (M)	O_2 uptake (% of control)	Inorganic P uptake (% of control)	Concn. of 2:4-dinitrophenol (M)	O_2 uptake (% of control)	Inorganic P uptake (% of control)
		β-Hydroxybutyrate oxidation			
$5{\cdot}3 \times 10^{-5}$	42	0	$4{\cdot}15 \times 10^{-5}$	100	13
$1{\cdot}76 \times 10^{-5}$	96	31	$1{\cdot}4 \times 10^{-5}$	100	52
$5{\cdot}8 \times 10^{-6}$	100	88	—	—	—
		Succinate oxidation			
$5{\cdot}3 \times 10^{-5}$	86	17	$3{\cdot}3 \times 10^{-5}$	81	4
$1{\cdot}76 \times 10^{-5}$	93	34	—	—	—
$5{\cdot}8 \times 10^{-6}$	95	87	—	—	—
		Citrate oxidation			
$2{\cdot}65 \times 10^{-5}$	52	16	$3{\cdot}3 \times 10^{-5}$	77	0
$1{\cdot}76 \times 10^{-5}$	55	33	$1{\cdot}1 \times 10^{-5}$	70	59
$5{\cdot}8 \times 10^{-6}$	88	68	—	—	—

workers have shown that lewisite and arsenite react readily with dithiols such as BAL to form stable five-membered ring structures (Stocken & Thompson, 1946*b*). Peters (1948), in his 'dithiol theory', suggested that arsenite and lewisite inhibited enzymes, the α-keto acid oxidases, which contain dithiol groups. This view has been confirmed by the demonstration of lipoic acid in purified pyruvic oxidase (Schweet & Cheslock, 1952) and α-oxoglutaric oxidase (Sanadi, Littlefield & Bock, 1952). The results given in this paper leave no reason to doubt that diethyltin dichloride could act in the same way as arsenite, phenylarsenious acid and lewisite. Some of our results with citrate as substrate (Fig. 1) suggest that for a comparable inhibition of oxygen uptake less α-oxoglutarate accumulates with diethyltin dichloride than with phenylarsenious acid. Sufficient results are not yet available to substantiate this suggestion, but if true it would suggest that diethyltin dichloride has other actions at the concentrations which inhibit α-oxoglutaric oxidase.

Diphenylchloroarsine possesses considerable affinity for monothiols and is a potent inhibitor, along with other SH reagents of *iso*citric dehydrogenase (Lotspeich & Peters, 1951). This inhibition is prevented by GSH. Diphenylchloroarsine inhibits oxidations of the tricarboxylic acid cycle and produces a slight accumulation of α-keto acid with citrate and L-malate as substrate and no change with L-glutamate. The mechanism of inhibition of oxidation by diphenylchloroarsine is not known and our results produce no new ideas. However, the effect of diphenylchloroarsine is quite unlike that of triethyltin sulphate. Triethyltin sulphate possesses little or no affinity for the SH groups of BAL or GSH. With rat-brain brei or with rat-liver mitochondria a consistent lowering of α-keto acid levels is found in the presence of triethyltin sulphate. The various steps of the respiratory chain are unaffected by approximately 100 times the concentrations effective against oxidation. Triethyltin sulphate has finally been shown to be a highly specific inhibitor of phosphorylation processes associated with oxidation. This finding poses many problems, of which three may be mentioned: (1) What is the mechanism of the inhibition of the phosphorylation process by triethyltin sulphate? (2) How does triethyltin sulphate interfere with oxidative processes? (3) Is the inhibition of phosphorylation found *in vitro* directly related to the clinical symtoms *in vivo*?

Practically nothing is known about the mechanism whereby energy derived from oxidation is converted into the phosphate bonds of ATP. The hypothesis that the triethyltin ion may replace some essential ion for the phosphorylation mechanism may be considered. Other substances, such as the SH reagents arsenite (Lehninger, 1949), iodoacetate and iodosobenzoate (Lehninger, 1949; Rothschild, Cori & Barron, 1954) and *p*-chloromercuribenzoate (Lehninger, 1951), are known to prevent oxidative phosphorylation. Triethyltin sulphate, however, possesses little affinity for SH groups. Magnesium is generally accepted as an essential element for oxidative phosphorylation (Lehninger, 1949; Lardy, 1951). Barkulis & Lehninger (1951) have shown that high concentrations of fluoride are inhibitory when AMP but not when ADP is used as a phosphate acceptor. They suggest that ADP is the actual phosphate acceptor and that the inhibition by fluoride when AMP is used is due to inhibition of myokinase which requires magnesium (Kalckar, 1943). If this is so, an inhibition of myokinase when AMP is used as phosphate acceptor will result in a prevention of phosphorylation. This cannot be an explanation for the action of triethyltin sulphate, since the same inhibition of phosphorylation is produced with either ATP or AMP. ATP in the presence of hexokinase and glucose is converted into ADP which acts as the phosphate acceptor.

It has been pointed out that several SH reagents inhibit oxidative phosphorylation. These substances are far from specific and inhibit oxidative enzymes at similar or lower concentrations. We have been unable to show a direct action of triethyltin sulphate on any oxidative step and the respiratory chain is not affected. However, inhibition of oxygen uptake is a striking biochemical effect of triethyltin sulphate, but inhibition of phosphorylation always occurs first. It seems reasonable to assume that the inhibition of oxidation is a consequence of the inhibition of phosphorylation. 2:4-Dinitrophenol also inhibits phosphorylation at lower concentrations than those which inhibit oxidation, but the margin between these two effects is larger than for triethyltin sulphate. The results of the experiments using β-hydroxybutyrate as substrate suggest that one consequence of stopping or lowering the rate of phosphorylation is a loss of Co I. However, the interconversion of Co I and ATP should not be forgotten (Kornberg, 1950; Judah, 1951). The rate of oxygen uptake is not maintained in the presence of Co I, but this may be due to further loss of the added Co I; the experiments were carried out in the presence of nicotinamide, but it is not known whether Co I can be lost under these circumstances by a mode of degradation not preventable by nicotinamide. With more organized oxidation systems, such as the tricarboxylic acid cycle, other phosphorylated coenzymes are required. It could be proposed that with triethyltin sulphate, as with a medium without AMP, the oxidizing ability is lost by a loss of these coenzymes. However, in the presence of

triethyltin sulphate, or in the absence of AMP, succinate oxidation is unimpaired. The prosthetic group of succinic dehydrogenase is not known with certainty (Neufeld, Scott & Stotz, 1954), but it is certainly stable and not easily separated from the enzyme protein.

Stoner *et al.* (1955) have described in detail the clinical symptoms produced by injection of triethyltin sulphate into rats. With a dose of 10 mg./kg. body wt. the most characteristic symptom is generalized muscular weakness which persists until they die 3–4 days later. At higher doses this weakness is much more pronounced and the animals are comatose. A consistent pathological finding has been an oedema of the central nervous system (Magee, Stoner & Barnes, unpublished). There appears to be no appreciable concentration of tin in any organ (Stoner *et al.* 1955); therefore, assuming that the triethyltin ion is distributed equally throughout the body water (75% of body wt.), an LD_{50} dose of 6 mg./kg. body wt. would give a concentration of 3×10^{-5}M. Upon this basis the inhibitory power of triethyltin sulphate on phosphorylation is sufficient to account for its toxicity. The symptoms are believed to have their origin in the central nervous system, and it should not be forgotten that most of our detailed experiments have been with liver mitochondria. However, our experiments with brain brei show that its inhibitory effect upon oxidation, which is presumably secondary to its effect on phosphorylation, occurs at similar concentrations to that on liver mitochondria. In view of the recent findings that many of the barbiturates inhibit oxidative phosphorylation (Brody & Bain, 1951, 1954; Bain, 1952), it is tempting to suggest that the narcotic effects of barbiturates and the generalized muscular weakness produced by triethyltin sulphate have a common origin in the inhibition of oxidative phosphorylation. The statement by Bain (1952) that barbiturate anaesthesia is potentiated by 2:4-dinitrophenol and that the latter drug is itself a depressant is clearly of great interest in this connexion.

SUMMARY

1. Diethyltin dichloride and triethyltin sulphate are powerful metabolic poisons and have different modes of action.

2. Diethyltin dichloride, like phenylarsenious acid, inhibits α-keto acid oxidases.

3. Triethyltin sulphate is a potent inhibitor of the phosphorylation processes associated with oxidation. No other action of triethyltin sulphate has been found.

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[^{14}C]Formate Labelling of Bases of Nucleic Acids in Respiring Slices of Rat Tissues

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Alterations in the physiological status of small laboratory animals (e.g. by hormone administration, or by exposing them to a cold environment) frequently cause well-defined changes in the subsequent metabolism of tissue slices taken from certain organs. As part of a general study of such effects, it was of interest to investigate the labelling of nucleic acids in tissue slices. Because of the importance of formate as a precursor of purines (reviewed by Brown, Roll & Weinfeld, 1952; Buchanan, 1952; Elwyn, 1953; Schulman, 1954), it was decided to investigate the possible incorporation of [^{14}C]formate into the bases of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in respiring slices.

Strangely enough, although there is a vast literature on the incorporation of small molecules into proteins, fatty acids and cholesterol in tissue slices, the nucleic acids have been studied less intensively. Much work has been done, however, on the formation of hypoxanthine in pigeon-liver preparations (summarized by Schulman, 1954).

Labelled inorganic phosphate is incorporated into the RNA of respiring slices of brain (DeLuca, Rossiter & Strickland, 1953) and of pancreas (Hokin & Hokin, 1954). In experiments of this type, most RNA preparations are contaminated with small amounts of highly radioactive phosphorus-containing compounds. For this reason, an unequivocal incorporation of the label into RNA had to await the development of adequate analytical methods (Davidson, Frazer & Hutchinson, 1951; Davidson & Smellie, 1952; DeLuca *et al.* 1953). With ^{14}C-labelled precursors, the earlier experiments were more convincing. Abrams, Goldinger & Barron (1950) showed that both RNA and DNA of bone-marrow slices were labelled from [*carboxy*-^{14}C]acetate. However, since Bernstein (1952) reported that [*carboxy*-^{14}C]acetate was incorporated into the ribose of RNA, it is not certain whether the bases or the sugars of the nucleic acids were labelled in these experiments. Reichard & Bergstrom (1951) showed that [^{15}N]glycine was incorporated into the purines of RNA and DNA of slices of regenerating liver, and, using a similar preparation, Lagerkvist, Reichard & Ehrensvärd (1951) and Reichard & Lagerkvist (1953) showed that labelled aspartic acid was incorporated into orotic acid and RNA pyrimidines. LePage (1953) reported that the purines of RNA and DNA were labelled from [α-^{14}C]glycine in liver and tumour preparations. Using bone-marrow slices, Abrams & Goldinger (1951) found that [*carboxy*-^{14}C]glycine and [^{14}C]formate (Abrams & Goldinger, 1952) were incorporated into the purines of both RNA and DNA. Totter (1954) showed that, in addition to the

purines, the methyl group in the 5-position of DNA thymine was also strongly labelled. As far as the present authors are aware, tissue-slice studies with [^{14}C]formate have been confined to bone marrow.

METHODS

Either male rats of the Sprague–Dawley strain or white guinea pigs were used. The animal was killed by decapitation. The liver, while still in position, was perfused with cold 0·154 M-NaCl through the hepatic vein. It was then removed, blotted with filter paper and cooled on ice. When required, other tissues were dissected out, blotted free from excess of blood and cooled on ice. Slices (total weight 400 mg. wet wt./flask) were prepared free-hand and placed directly into tared Warburg vessels containing 4·5 ml. saline buffered at pH 7·4 (either Krebs–Ringer bicarbonate or Krebs–Ringer phosphate solution). Each flask was then reweighed and sufficient sodium [^{14}C]formate dissolved in 500 μl. buffered Ringer solution was added to give 6·75 μ-moles and 15×10^6 counts/min. per flask. In experiments with inhibitors, the inhibitor was made up in buffered Ringer solution. The final volume was maintained at 5 ml. The flasks were then gassed with the appropriate gas mixture [$O_2 + CO_2$ (95:5) for bicarbonate buffer, O_2 for phosphate buffer]. In anaerobic experiments N_2 replaced O_2, and the gas mixture was passed over heated Cu filings to remove the last traces of O_2. The vessels were then incubated at 37·4 $\pm$ 0·1° for 4 hr., with shaking to ensure proper oxygenation.

At the end of the incubation period the suspending medium was decanted and the tissue was transferred, with the addition of small portions of cold 95% (v/v) ethanol, to a glass tube and homogenized with an electrically driven pestle. The homogenate was centrifuged and the supernatant discarded. Further lipid was extracted from the residue by successive treatment with 5 ml. portions of cold ethanol (twice), 3:1 (v/v) ethanol–ether mixture heated to boiling for 3 min. (three times), and finally ether at room temperature. The residue was extracted at 2–4° with 10% (w/v) trichloroacetic acid (TCA), followed by two further extractions with 10% TCA containing carrier formate (0·05 M-H.CO_2Na). For the second and third extractions, the tissue residue was suspended in 2·5 ml. distilled water and N-NaOH was added drop by drop until the precipitate dissolved. To this solution was added 2·5 ml. 20% TCA–carrier formate mixture. The precipitate was centrifuged and the supernatant poured off. The residue was finally extracted with 10% TCA containing no carrier formate. This latter extract was free from radioactivity.

Nucleic acids were extracted from the residue by the method of Hammarsten (1947). The dried nucleic acid precipitate was weighed out in small test tubes and hydrolysed with 0·3 N-KOH at 37° for 18 hr., 50 μl. alkali per mg. nucleic acid being used. The nucleic acids were separated into DNA and RNA nucleotides by the method of Schmidt & Thannhauser (1945). The purine and pyrimidine bases of both DNA and RNA were liberated by hydrolysis with 72% (w/v) $HClO_4$, as described by Marshak & Vogel (1951). The bases were isolated by the method of paper chromatography described by Wyatt (1951). Spots were located by viewing the dried chromatogram in ultraviolet light (Mineralight) against a fluorescent background. The bases were identified by their positions relative to those of a mixture of authentic samples of purines and pyrimidines. The spots were outlined in pencil and a circular area of filter paper 3 cm. in diameter was punched out from the centre of each of the spots. Each of the paper circles was weighed so that a correction could be made for absorption of β-particles due to the filter paper, as described by LePage & Heidelberger (1951). The radioactivity was determined by placing the paper circle in a gas-flow internal counter (Nuclear Instrument and Chemical Corp., Chicago). The circle was then cut into small pieces and eluted for at least 3 hr. in a large test tube with 10 ml. 0·1 N-HCl. The ultraviolet absorption of the eluted base was determined at $\lambda_{max.}$ by means of a Beckman model DU spectrophotometer. The concentration of base in the eluate was calculated from the molecular extinction coefficients given in Table 1. The values found for $\lambda_{max.}$ agreed well with the values quoted in the literature. The specific activity, in terms of counts/min./μmole, was calculated for each base.

Table 1. *Absorption data for nucleic acid bases*

Base	$\lambda_{max.}$	ϵ	Reference
Adenine	265	13 100	Loring, Fairley, Bortner & Seagran (1952)
Guanine	250	11 000	Loring *et al.* (1952)
Uracil	260	8 130	Ploeser & Loring (1949)
Cytosine	275	10 140	Ploeser & Loring (1949)
Thymine	265	7 940	Vischer & Chargaff (1948)

RESULTS

Labelling of RNA purines of guinea-pig and rat liver

Table 2 shows that the radioactivity of the labelled formate was incorporated into both the adenine and the guanine of the RNA of liver slices from both guinea pig and rat. In these experiments the slices were incubated for 4 hr. in Krebs–Ringer bicarbonate solution buffered at pH 7·4. The table also shows that for rat liver the ratio of the specific activity of adenine to that of guanine was much greater than that observed for guinea-pig liver.

Table 2. *Labelling of adenine and guanine in RNA of slices of guinea-pig and rat liver incubated with* [^{14}C]*formate in Krebs–Ringer bicarbonate solution*

	No. of animals	Specific activity (counts/min./μmole. Mean $\pm$ S.E.M.) Adenine	Guanine	Ratio, adenine/guanine
Guinea pig	4	1750 $\pm$ 90	1050 $\pm$ 34	1·7
Rat	6	3400 $\pm$ 240	540 $\pm$ 100	6·3

The experiments reported in Table 3 show that at pH 7·4 the RNA purines of rat-liver slices were labelled from [^{14}C]formate in a medium buffered either with bicarbonate or with phosphate. With phosphate buffer the incorporation of the 'label' into adenine appeared to be slightly less than with

bicarbonate, but the incorporation into guanine was greater. Table 3 also illustrates the degree of reproducibility obtained in experiments of this type when duplicate samples were taken from the same liver.

Table 3. *Effect of buffer on the labelling of adenine and guanine in RNA of slices of rat liver incubated with* [^{14}C]*formate in Krebs–Ringer solution at* pH 7·4.

	Specific activity (counts/min./μmole)			
	Adenine		Guanine	
Expt. no.	Bicarbonate buffer	Phosphate buffer	Bicarbonate buffer	Phosphate buffer
1	3200	2300	470	620
	2700	2300	420	590
2	4500	3200	800	970
	4300	3300	870	1070

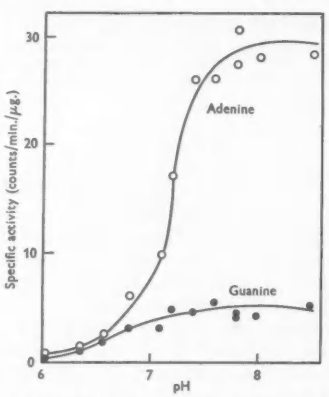

Fig. 1. Effect of pH on the labelling of adenine and guanine in RNA of slices of rat liver incubated with [^{14}C]formate in Krebs–Ringer solution.

By using phosphate buffer, the effect of the pH of the incubating medium was investigated for the RNA purines of rat-liver slices (Fig. 1). The extent of the labelling of both purines increased with increasing pH, up to 7·5. Beyond this the effect of pH was less marked. Good labelling was still observed at pH 8·5. The effect of changing the pH was greater for adenine than for guanine. When the pH was less than 6·5, the labelling of the two purines was similar, but at higher pH values more of the label was incorporated into adenine. In many of the subsequent experiments phosphate buffer was used at pH 7·8.

The high optimum pH for the labelling of the RNA purines in rat-liver slices is in contrast to the low pH of 6·5, reported by Curran (1953) to be optimum for the labelling of cholesterol and fatty acid from [^{14}C]acetate in similar preparations. That such a low pH gives optimum cholesterol and fatty acid labelling has been confirmed in this Laboratory.

Fig. 2 shows the time course of the labelling of RNA purines of rat-liver slices. After an initial lag period, the incorporation was linear for 2 hr., after which there appeared to be a slight falling-off. At the end of 4 hr. the ratio of the activity of adenine to that of guanine was greater than the ratio found at the end of 2 hr.

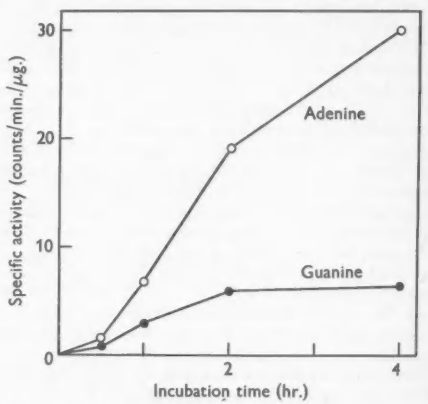

Fig. 2. Time course of the labelling of adenine and guanine in RNA of slices of rat liver incubated with [^{14}C]formate in Krebs–Ringer solution.

Table 4. *Effect of metabolic inhibitors on the labelling of adenine and guanine in RNA of slices of guinea-pig liver incubated with* [^{14}C]*formate in Krebs–Ringer bicarbonate solution at* pH 7·4

		Specific activity (counts/min./μmole)	
Expt. no.	Inhibitor added	Adenine	Guanine
1	None	1010	580
	None (zero-time sample*)	0	0
2	None	1900	780
	KCN (0·03 M)	0	0
3	None	1600	1300
	2:4-Dinitrophenol (10^{-4} M)	180	100
	None (anaerobic incubation)	0	0

* Control experiment without incubation (see text).

Metabolic inhibitors

Table 4 shows the effect of metabolic inhibitors on the labelling of RNA purines of guinea-pig liver slices. In Expt. 1 the zero-time sample represents

slices that were prepared in the usual manner, placed in the medium containing radioactive formate, and then homogenized in ethanol without incubation. A figure of 0 is reported if the counting rate of the isolated base was less than one-half of the background counting rate.

Expt. 2 shows that the incorporation into both purines was abolished in the presence of 0·03M-KCN. Expt. 3 shows that there was no incorporation if the conditions were anerobic and that the incorporation was reduced greatly in the presence of 2:4-dinitrophenol (10^{-4}M).

Other tissues

The labelling of RNA purines was determined in slices from a variety of rat tissues. In the two experiments reported in Table 5 the descending order of activity for both adenine and guanine was: spleen, liver, kidney, pancreas, brain, heart. In general, the order of activity was similar to that reported by Drochmans, Marrian & Brown (1952) and Bendich, Russell & Brown (1953) for the RNA purines of corresponding tissues after the administration of [^{14}C]formate. The most notable difference was that, in the *in vivo* experiments, kidney RNA purines were more strongly labelled than those of liver or spleen.

The figures quoted for spleen deserve some comment. In the spleens of the two animals used for the experiments reported in Table 5 and in the spleens of two further animals, used in the experiments reported in Table 7, the incorporation of the radioactivity from formate was of a high order. However, in a number of animals studied subsequently, the labelling of the RNA purines of spleen slices, although still greater than the labelling of the RNA purines of liver slices, was less than that reported in Table 5.

The ratio of the specific activity of adenine to that of guanine was less for rat spleen than for most other rat tissues. In this regard rat spleen resembled guinea-pig liver (Table 2).

Labelling of DNA purines and pyrimidines

In certain of the experiments with slices of rat liver (Table 6) or spleen (Table 7), the labelling of the four bases of RNA and the four major bases of DNA was studied. In liver slices (Table 6) RNA adenine was most strongly labelled, followed by DNA adenine. The RNA guanine and DNA guanine were next in order. Cytosine and uracil were less well labelled, but the incorporation into thymine was of the same order as the incorporation into DNA guanine. Each of the RNA purines was more strongly labelled than the corresponding DNA purines, but this difference was much greater for adenine than for guanine.

For spleen slices (Table 7), the incorporations were all much greater. Again, adenine was more strongly labelled than guanine and the RNA purines were more strongly labelled than the DNA purines. Each of the pyrimidines demonstrated definite radioactivity, the activity of thymine being of the same order as that of DNA guanine.

Table 5. *Labelling of adenine and guanine in RNA of slices of rat tissue incubated with* [^{14}C]*formate in Krebs–Ringer phosphate solution at* pH 7·8

	Specific activity (counts/min./μmole)				Ratio, adenine/guanine	
	Adenine		Guanine			
Tissue	Rat A	Rat B	Rat A	Rat B	Rat A	Rat B
Spleen	26 300	21 900	11 200	11 300	2·3	1·9
Liver	4 900	4 800	1 200	980	4·1	4·9
Kidney	3 000	2 300	590	480	5·1	4·8
Pancreas	820	500	170	120	4·8	4·2
Brain	260	300	60	60	4·3	5·0
Heart	110	55	30	30	3·7	1·8

Effect of fasting for 24 hr.

In the rat, deprivation of food greatly decreases the labelling of fatty acids (Masoro, Chaikoff, Chernick & Felts, 1950; Medes, Thomas & Weinhouse, 1952) and cholesterol (Tomkins & Chaikoff, 1952) from [^{14}C]acetate in liver slices. Table 8 shows that a fast of 24 hr. caused no similar change in the labelling of RNA purines from [^{14}C]formate.

Hypophysectomy

Brady, Lukens & Gurin (1951) reported that hypophysectomy increased the labelling of fatty acids from [^{14}C]acetate in liver slices and Tomkins, Chaikoff & Bennett (1952) reported a decrease in the labelling of cholesterol. Table 9 shows that removal of the pituitary had no significant effect on the labelling of RNA adenine from [^{14}C]formate. These experiments were performed 14 days after the removal of the pituitary. Other experiments

Table 6. *Labelling of purines and pyrimidines of RNA and DNA of slices of rat liver incubated with* [^{14}C]*formate in Krebs–Ringer phosphate solution at* pH 7·8

	Specific activity (counts/min./μmole)					
	RNA		DNA		Ratio, RNA/DNA	
Base	Rat C	Rat D	Rat C	Rat D	Rat C	Rat D
Adenine	5200	6300	1300	1000	4·0	6·3
Guanine	830	830	710	420	1·2	2·0
Cytosine	190	460	290	530	—	—
Uracil	340	540	—	—	—	—
Thymine	—	—	810	550	—	—

Table 7. *Labelling of purines and pyrimidines of RNA and DNA of slices of rat spleen incubated with* [^{14}C]*formate in Krebs–Ringer phosphate solution at* pH 7·8

	Specific activity (counts/min./μmole)					
	RNA		DNA		Ratio, RNA/DNA	
Base	Rat C	Rat D	Rat C	Rat D	Rat C	Rat D
Adenine	25 700	20 100	11 700	6 600	2·2	3·0
Guanine	18 000	15 600	9 500	6 800	1·9	2·3
Cytosine	1 700	1 700	510	400	—	—
Uracil	970	990	—	—	—	—
Thymine	—	—	6 800	5 000	—	—

Table 8. *Effect of fasting for* 24 *hr. on the labelling of adenine and guanine in RNA of slices of rat liver incubated with* [^{14}C]*formate in Krebs–Ringer bicarbonate solution at* pH 7·4

	No. of animals	Specific activity (counts/min./μmole, mean ± S.E.M.)	
		Adenine	Guanine
Fed *ad lib.*	4	2600 ± 300	610 ± 100
Fasted 24 hr.	6	2700 ± 270	710 ± 70

Table 9. *Effect of hypophysectomy and a sham operation on the labelling of adenine in RNA of slices of rat liver incubated with* [^{14}C]*formate in Krebs–Ringer bicarbonate solution at* pH 7·4

The animals were studied fourteen days after operation.

	No. of animals	Specific activity (counts/min./μmole, mean ± S.E.M.)
Normal	6	3300 ± 340
Hypophysectomized	11	4000 ± 330
Sham operation	6	4100 ± 390

showed that 2 days after hypophysectomy there was no significant change in the labelling of RNA adenine.

DISCUSSION

The experiments reported in Table 4 on the inhibitory effect of cyanide and 2:4-dinitrophenol provide good evidence that the labelling of the RNA purines is a metabolic phenomenon and that it is not a simple exchange reaction. The inhibition caused by 2:4-dinitrophenol (10^{-4} M) and the complete absence of labelling under anaerobic conditions strongly suggest that the incorporation is dependent upon the production of energy-rich compounds by oxidative phosphorylation. A similar situation is encountered in the labelling of the nucleotides of RNA with inorganic ^{32}P in brain slices (DeLuca *et al.* 1953; Findlay, Rossiter & Strickland, 1953). In this regard, it is of interest to note that Abrams *et al.* (1950) found that the incorporation of [*carboxy*-^{14}C]acetate into the RNA and DNA of bone-marrow slices was inhibited by anaerobic conditions and Goldwasser (1953) found that the incorporation of [^{14}C]adenine into the RNA of liver slices was inhibited by 3×10^{-3} M-KCN.

The general pattern observed for slices of liver and spleen—good labelling of the purines of RNA, with lesser labelling of the pyrimidines, and good labelling of the purines and thymine of DNA, with lesser labelling of cytosine—is in agreement with the *in vitro* experiments of Totter (1954) for bone marrow. Previously, a similar pattern was reported for various rat tissues after the injection of [^{14}C]formate (Heinrich & Wilson, 1950; Totter, Volkin & Carter, 1951; Bendich *et al.* 1953). In experiments of this type, the nucleic acid purines were found to be labelled in positions 2 and 8 (Heinrich & Wilson, 1950; Drysdale, Plaut & Lardy, 1951) and the thymine was labelled in the methyl group at position 5 (Totter *et al.* 1951).

The finding that adenine was labelled more strongly than guanine in RNA is in agreement with the results of *in vitro* studies with bone-marrow

slices, in which either [*carboxy*-^{14}C]glycine (Abrams & Goldinger, 1951) or [^{14}C]formate (Abrams & Goldinger, 1951; Totter, 1954) was the source of the label. In the experiments reported here with liver slices, the ratio of the activity of RNA adenine to that of RNA guanine was found to depend upon the organ studied (Table 5), the species (Table 2), the nature of the buffer (Table 3), the pH (Fig. 1) and the time of the incubation (Fig. 2). It is not surprising, therefore, that with *in vivo* experiments great differences are reported in the literature for this ratio. In such experiments a wide variety of species, tissues, injection times etc. were used.

The finding that for both liver and spleen slices the labelling of RNA adenine and guanine was greater than that of the corresponding DNA purine is similar to previous *in vivo* experiments, in which liver and spleen were studied after the administration of [^{14}C]formate (Bendich *et al.* 1953; Smellie, McIndoe & Davidson, 1953). *In vivo* experiments with ^{32}P, in which animals were killed at short intervals after administration of the isotope, showed that the labelling of RNA was considerably greater than the labelling of DNA, for tissues not rapidly proliferating (Brues, Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Volkin & Carter, 1951; Davidson & Smellie, 1952). In tissue-slice experiments with ^{32}P, RNA is much more strongly labelled than DNA. Indeed, because of the possibility that non-radioactive DNA may be contaminated with traces of much more radioactive RNA, *in vitro* studies with ^{32}P, such as those of DeLuca *et al.* (1953) with brain slices and Kit, Bacila & Barron (1954) with lymphocytic cells, make it uncertain whether DNA is labelled at all. This would be in conformity with the suggestion that ^{32}P is incorporated into DNA only during cell division (Marshak, 1941; Hull & Kirk, 1950; Osgood, Li, Tivey, Duerst & Seaman, 1951; Stevens, Daoust & Leblond, 1953).

The figures reported in Tables 6 and 7 indicate that, unlike *in vitro* studies with ^{32}P, DNA was strongly labelled when the radioactive precursor was [^{14}C]formate. If it were assumed that the DNA purines were not labelled at all and that the radioactivity observed was due to contamination of non-radioactive DNA with radioactive RNA, the contamination would have to be gross (more than 50% in some instances). Such gross contamination would be detected by the presence of uracil in the chromatograms. Also, the labelling of thymine could not be explained in terms of contamination of the DNA with RNA. The *in vitro* labelling of DNA purines in bone-marrow slices from [^{14}C]acetate (Abrams *et al.* 1950), [^{14}C]glycine (Abrams & Goldinger, 1951) and [^{14}C]formate (Abrams & Goldinger, 1952; Totter, 1954) has been reported.

Alteration of the physiological status of the animal would appear to have little effect on the subsequent ability of liver slices to label RNA purines. This is illustrated by the findings that food deprivation for 24 hr. (Table 8) or removal of the pituitary gland (Table 9) caused no significant change. In addition, it was found that the presence of insulin *in vitro*, either with or without the addition of glucose, had no significant effect on the labelling of RNA purines in rat-liver slices (unpublished experiments). Exposing the animals to an environmental temperature of 3° for periods of time ranging from 3 hr. to 28 days, or exposing them for 3 hr. to temperatures ranging from 5° to −10°, also had no consistent significant effect on the labelling of the RNA or DNA purines of liver or spleen slices. These findings are in sharp contrast to the labelling of cholesterol (Tomkins & Chaikoff, 1952; Tomkins *et al.* 1952), fatty acid (Masoro *et al.* 1950; Brady *et al.* 1951; Medes *et al.* 1952) and phospholipid (Rossiter & Kline, 1955) of liver slices from [^{14}C]acetate. The degree of labelling of these substances is influenced greatly by the preceding physiological condition of the animal.

SUMMARY

1. When slices of rat liver or spleen were allowed to respire in Krebs–Ringer solution containing [^{14}C]formate, radioactivity was detected in the adenine and guanine of RNA and the adenine, guanine and thymine of DNA. The labelling of the RNA purines of liver took place when the slices were incubated in either phosphate or bicarbonate buffer. Optimum labelling occurred at a pH greater than 7·5.

2. Under similar conditions, the purines of the RNA of slices of guinea-pig liver were labelled. The labelling was inhibited by KCN (0·03 M), 2:4-dinitrophenol (10^{-4} M), or by rendering the conditions anaerobic.

3. Adenine and guanine of RNA in slices of other organs were also labelled. For the rat, the descending order of activity was: spleen, liver, kidney, pancreas, brain, heart.

4. Labelling of the purines of RNA was not affected by a previous fast of 24 hr. or by removing the pituitary gland.

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Formation and Properties of the Activator of Plasminogen and of Human and Bovine Plasmin

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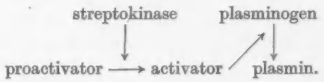
Plasminogen is the precursor of plasmin, a proteolytic and fibrinolytic enzyme of blood. Streptokinase, an agent produced by cerlain streptococci (Garner & Tillett, 1934), effects the conversion of human plasminogen into plasmin (Kaplan, 1944; Christensen, 1945), presumably by a direct enzymic effect (Christensen & MacLeod, 1945). Bovine plasminogen was activated only very slightly by streptokinase, but large amounts of plasmin were produced in bovine plasminogen by a tissue activator (Astrup & Permin, 1948), probably by a stoicheiometric reaction (Astrup, 1951). Geiger (1952) demonstrated an increased proteolytic activity in mixtures of globulin preparations from man and other species after activation by streptokinase. He suggested that a complement-like factor was involved in the activation of plasminogen by streptokinase. Müllertz & Lassen (1953)

showed that large quantities of bovine plasmin were formed on the addition of a mixture of streptokinase and human globulin to bovine plasminogen, and that large amounts of an activator of plasminogen were formed by the addition of streptokinase to human globulin. The demonstration of an activator of plasminogen in fibrinolytic human blood (Müllertz, 1953) indicated that all components necessary for formation of the activator were present in blood. It was suggested (Müllertz, 1952; Müllertz & Lassen, 1953) that a proactivator–activator system was involved in the activation of plasminogen in blood. In the following scheme the interaction of the different components is shown, but the type of the reactions is not denoted:



As bovine plasminogen preparations contain large amounts of plasminogen, and little or no proactivator, bovine plasmin is not formed on addition of streptokinase. Human blood contains large amounts of proactivator and relatively small amounts of plasminogen. By addition of streptokinase large amounts of activator and small amounts of plasmin are formed.

In the present paper methods for the estimation of activator and of plasmin are described, and the formation of activator by the interaction of streptokinase and proactivator is studied. By means of the activator a complete activation of bovine plasminogen is obtained. Properties of the activator and of partially purified, completely activated and activator-free preparations of bovine and human plasmin are described and compared.

MATERIALS AMD METHODS

Human globulin. Serum was obtained from out-dated, citrated, pooled bank blood by recalcification. The euglobulin fraction was precipitated by diluting the serum twenty times with distilled water and adding 1% acetic acid until a pH value of 5·3 was obtained. The precipitate was dissolved in 0·9% NaCl (adding 0·1 N-NaOH until a pH value of 7·6 was reached) or phosphate buffer (pH 7·4 or 7·6) to a volume equal to 20% of the original serum volume. The solution was stored at −20°. A single stock solution of globulin was used in the experiments on the activator, and contained 2·9 mg. nitrogen/ml. (micro-Kjeldahl). The concentration of human globulin was expressed as the volume of this stock solution (in ml.) contained in 1 ml. of the final solutions (= ml./ml.).

Streptokinase. Varidase (kindly supplied by Lederle Laboratories Division) was used. It was a lyophilized preparation containing about 3000 units/mg. A stock solution containing 10 mg./ml. distilled water was stored at −20°.

Bovine plasminogen. The preparation and properties were as described by Müllertz (1953) and Müllertz & Lassen (1953). No significant activity on casein was present or could be produced by streptokinase in solutions of this preparation.

Plasminogen activator. Human globulin (4 vol.) and streptokinase (10 mg./ml., 1 vol.) were incubated at 37° for 10 min., cooled and diluted with ice-cold phosphate buffer (pH 7·4 or 7·6). A maximal conversion of proactivator into activator was obtained. The final concentration of activator was expressed as per cent (v/v) of the undiluted solution prepared fresh each time.

Bovine plasmin. A solution of plasminogen (5 mg./ml.) containing 1% (v/v) activator was left for 4 hr. at 22° and pH 7·4 to obtain a complete activation. The solution was adjusted to pH 2·9 by addition of 0·15 N-HCl, left at 22° for 30 min. and brought to pH 7·6 by addition of 0·15 N-NaOH. This treatment was found to destroy activator in the presence of plasminogen with only a small loss of plasmin. The final concentration was equivalent to 2·5 mg. of the plasminogen preparation/ml. The activity of the human plasmin added with the activator solution was about 3% of the total activity. The bovine plasmin had approximately the same activity as the human plasmin and contained about half as much nitrogen per ml. (0·13 mg. N/ml., micro-Kjeldahl).

Human plasmin. Human globulin (100 ml.) was mixed with 100 ml. of 0·9% NaCl and 50 ml. of streptokinase (0·2 mg./ml.) and kept at 37° for 15 min. A maximal formation of plasmin, as estimated by the heated fibrin plate method, occurred under these conditions. The solution was adjusted to pH 1·8 with 0·15 N-HCl, immersed in a boiling-water bath for 30 min., cooled and brought back to pH 7·6 and a volume of 500 ml. with 0·15 N-NaOH and phosphate buffer (pH 7·6). The destruction of inhibitory substances, of streptokinase and of activator by this treatment was established in separate experiments. Denatured protein was removed by centrifuging and the supernatant was dialysed against distilled water at 5° for 48 hr. and adjusted to pH 5·3 by addition of 1% acetic acid. After centrifuging, the precipitate was dissolved in 100 ml. of 0·9% NaCl, 0·15 N-NaOH being added to give a pH 7·4 or 7·6. The solution was stirred for 30 min. at 40° to achieve as complete a dissolution as possible, and undissolved protein was removed by centrifuging. The loss in activity could not be accurately determined because inhibitors were destroyed simultaneously with plasmin. The final solution contained 0·24 mg. N/ml. (micro-Kjeldahl) and was about seven times more active (per mg. N) than the original solution (heated fibrin plate method).

Buffers. Sodium diethylbarbiturate buffer (Michaelis) 0·1 M, pH 7·8, was used in the preparation of the fibrin plates. Phosphate buffer (Sørensen), 0·1 M, pH 7·6 (or 7·4), was used in the casein experiments. In the experiments on pH of optimum stability and optimum activity the following buffers were used: lactate buffer, 0·15 M, pH 2·9; acetate buffer, 0·15 M, pH 4·0–5·5; phosphate buffer (Sørensen) 0·1 M, pH 6·0–7·6; borate buffer (Sørensen), 0·1 M, pH 8·0–10·3. To the buffer solutions were added NaCl or distilled water so as to give an ionic strength of 0·15.

Casein. 'Hammarsten' casein (Merck) (3 g.) was dissolved in 100 ml. of distilled water by addition of 0·15 N-NaOH to pH 8·0, heated at 100° for 15 min. and cooled (Kunitz, 1947). As this preparation yielded high and varying blank values (at 275 mμ., after deproteinization), the casein was precipitated with perchloric acid. The amount

of low-molecular compounds adhering to the precipitate was reduced by precipitating at the acid side of the isoelectric point and by repeated washing. A boiled solution (100 ml.) of casein was acidified by the dropwise addition of 2–3 ml. of N-NCl, with constant stirring. At the onset of precipitation 4 ml. of N-HCl were added quickly, bringing the pH to 2·0–2·2. In this way a massive isoelectric precipitation of slowly soluble casein was avoided. The clear solution (100 ml.) was precipitated by the quick addition of 150 ml. of 0·17 M perchloric acid and left suspended in the acid overnight at room temp. It was washed three times with distilled water, redissolved in phosphate buffer and 0·15 N-NaOH and made up to the original volume, a pH of 7·4 or 7·6 and an ionic strength of 0·15. The solution, designated as 3% (w/v) casein, was stored in small bottles (10–20 ml.) at −20°. This solution produced blank values below 0·100 (at 275 mμ., after deproteinization, at 1·5%, w/v) and was completely stable for 20 min. at 37°.

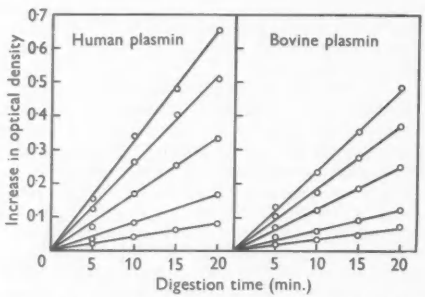


Fig. 1. Relationship between increase in optical density and digestion time for mixtures of equal volumes of casein (3·0%, w/v) and plasmin (100, 75, 50, 25, and 12·5%, v/v) (37°, pH 7·6).

Estimation of plasmin by casein digestion. Proteolytic splitting of casein was determined by the increase in optical density at 275 mμ. of the acid-deproteinized reaction mixtures (Kunitz, 1947). The absorption in the range 270–280 mμ. was found to be considerable for trichloroacetic acid, but insignificant for perchloric acid, which was preferred. A maximal precipitation of protein in 1·5% (w/v) casein solutions was obtained at final concentrations above 0·25 M perchloric acid. A final concentration of M perchloric acid was used and yielded reproducible and low readings with blank mixtures of casein and human and bovine plasmin.

The casein solution (3%, w/v) and the enzyme solution were placed in a water bath (37°) until temperature equilibrium was reached (5 min.) and equal volumes were then mixed in a test tube. Samples of 2 ml. were removed immediately after mixing and after 5, 10, 15 and 20 min. at 37°, and pipetted into test tubes containing 3 ml. of 1·7 M perchloric acid. After 1 hr. at room temp. the precipitates were removed by centrifuging. The optical densities of the supernatants were read against a water blank and the increase in optical density was calculated, 2 cm. cuvettes being used. Deproteinized solutions of casein digested by bovine plasmin showed an absorption maximum at 275 mμ. and all readings were done at this wavelength. A Hilger Uvispek spectrophotometer was used.

With human and bovine plasmin proportionality between increase in optical density E and digestion time t at a constant enzyme concentration was observed (Fig. 1). The velocity constant k, calculated as the increase in optical density per minute of digestion time, denotes the slope of the curves. Proportionality was also found between values of k and enzyme concentration (Fig. 2), indicating that k could be used as a correct expression of the enzyme concentration. Under the applied conditions the splitting of casein followed a zero-order reaction, indicating that the casein substrate was present in excess.

The standard deviation on k was found to be 1·9% (mean value, 0·0176; n, number of determinations, 14). In some experiments the increase in optical density per 20 min., $E_0 - E_{20}$, was determined by removing samples at 0 and 20 min. only. The standard deviation on $E_0 - E_{20}$ at

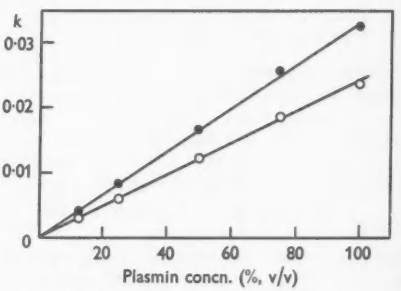


Fig. 2. Relationship between enzyme concentration and activity; k = increase in optical density/min. digestion time. ●, Human plasmin; ○, bovine plasmin (37°, pH 7·6).

three different enzyme concentrations was 2·7% (mean, 0·244), 4·7% (mean, 0·171) and 9·0% (mean, 0·078) (n = 14).

Estimation of plasmin by fibrinolysis. The heated fibrin plate method described by Lassen (1952) was used. The estimations were performed as described by Astrup & Müllertz (1952). The standard deviation on an estimation of human plasmin was determined to 5%.

Estimation of activator on a bovine-fibrin substrate containing plasminogen. Astrup & Permin (1947) originally demonstrated that purified bovine fibrinogen contains large amounts of plasminogen, and this observation has repeatedly been confirmed (Lewis & Ferguson, 1950; Lassen, 1952; Müllertz & Lassen, 1953; Sherry, 1954). 'Armour' bovine fraction I and bovine fibrinogen prepared as described by Ware, Guest & Seegers (1947) or Jaques (1943) all contain large quantities of plasminogen (Astrup, personal communication). Human globulin, activated by streptokinase, contains large amounts of activator and relatively small amounts of plasmin, and the activator was estimated from the lysis produced by activation of the plasminogen contained in the bovine-fibrin substrate of the standard fibrin plate method (Astrup & Müllertz, 1952). The standard deviation on a single estimation was 5%. The plasmin present in the highly diluted samples used for the

assay of the activator by this method produced no lysis of a plasminogen-free fibrin substrate (the heated fibrin plate method, Lassen, 1952). The susceptibility of unheated and heated fibrin to plasmin was determined on six different samples of activator-free human and bovine plasmin. Unheated fibrin was two to four times more susceptible to plasmin than heated fibrin, but this difference must be considered maximal, as even minute amounts of activator in the plasmin samples will cause increased lysis in the unheated fibrin substrate. Consequently, the lysis of diluted activator solutions in clotted bovine fibrinogen is caused by activation of plasminogen in the substrate, and the effect of the plasmin present in the samples is insignificant.

Estimation of activator by means of casein containing added bovine plasminogen. Activator was determined from its effect on an excess of bovine plasminogen in the presence of an excess of casein, as follows. In one test tube 1·00 ml. of a solution of bovine plasminogen (5 mg./ml.), 2·50 ml. of a solution of casein (3%, w/v), and 0·50 ml. of phosphate buffer (pH 7·6) were mixed, and 1·00 ml. of a solution of activator was added to another test tube. The tubes were incubated until temperature equilibrium was reached (5 min.) and the contents were mixed by pouring from one tube to the other several times. Samples of 2·00 ml. were removed immediately after mixing and after 20 min. at 37° and pipetted into test tubes containing 3·00 ml. of 1·7 M perchloric acid. After centrifuging, the optical density at 275 mμ. of the supernatants was determined and the increase per 20 min. ($E_0 - E_{20}$) was calculated.

A reference curve for activator concentration was obtained as follows: Solutions were prepared containing human globulin (0·40 ml./ml.) and 8·0. 1·6, and 0·8 mg. of streptokinase/ml. respectively. At 8·0 and 0·8 mg. of streptokinase/ml. a complete conversion of proactivator or of streptokinase respectively was obtained, and a concentration of streptokinase of 1·6 mg./ml. was found to be equivalent to the globulin concentration used. Appropriate dilutions of these solutions were prepared and the activator activity was determined as described. By plotting $E_0 - E_{20}$ against activator concentration, curves of identical shape were obtained with all preparations (Fig. 3). By means of this curve the activator content of a given solution can be expressed as a per cent of a standard solution or in arbitrary activator units. Controls of (1) casein + buffer, (2) casein + plasminogen, (3) casein + streptokinase and (4) casein + plasminogen + streptokinase showed no or negligible increases in optical density per 20 min. at 37°. The activity of the human plasmin added with the solutions was determined in a control of (5) casein + activator, and amounted to 1–2% of the total activity observed in the mixture of casein, plasminogen and activator. The accuracy of the method was determined in a series of experiments. In the experiments described in section (ii) single batches of casein and plasminogen, stored in solution at −20°, were used, and the standard deviation on a single estimation of activator was found to be 2·0% ($n = 10$) and 1·6% ($n = 10$). In the remaining experiments two lyophilized preparations of plasminogen and two deep-frozen batches of casein were used, and the standard deviation was found to be 5·6% ($n = 16$) and 4·9% ($n = 10$).

Method used to study the formation of activator. The components were allowed to react in relatively concentrated solutions, and the amount of activator formed was determined in highly diluted samples withdrawn from the activation mixtures. In the experiments described in section (ii), 25 μl. samples were removed and added to the assay mixture of casein and plasminogen, a final dilution of 1:200 being obtained. In the remaining experiments, the reaction mixtures were still more concentrated, and 25 μl. samples were removed and mixed with 3·00 ml. of phosphate buffer (37°), from which 1·00 ml. was immediately transferred to the assay mixture, a final dilution of 1:605 being obtained. A necessary precaution for a study of the formation of activator under varying conditions was that the reaction was not significantly influenced by the assay procedure. Information on this point was supplied by an estimation of the maximum amount of activator that might form during the assay. Solutions of globulin and of streptokinase were diluted separately, and added at the same time to a mixture of casein and plasminogen in concentrations equal to those used for the determinations described above. The amount of activator formed during the assay was expressed as a per cent of the amount which was formed by the same quantities of streptokinase and globulin when the reaction was allowed to run to completion in concentrated solutions (2 hr., 22°) before addition to the assay mixture. For concentrated solutions of human globulin (0·40 ml./ml.) and streptokinase (8 mg./ml.), and of human globulin (0·15 ml./ml.) and streptokinase (0·60 mg./ml.), 4 and 2% respectively were formed, and even smaller values were observed for other solutions. Thus the rate of reaction in diluted solutions was so slow that no significant change was likely to take place during

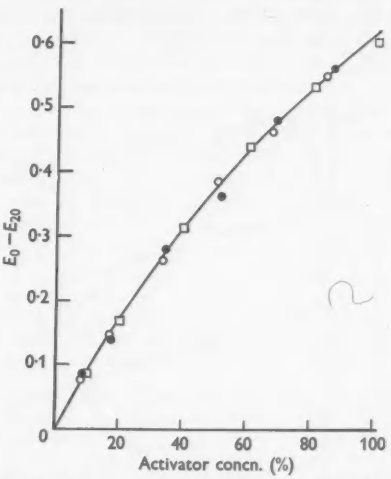


Fig. 3. A reference curve for activator concentration. Solutions containing: human globulin (0·40 ml./ml.); □, 8·0 mg.; ○, 1·6 mg.; ●, 0·8 mg. of streptokinase/ml.; were left at 22° for 4 hr. to complete the reactions and diluted in an ice bath to obtain stock solutions of equal activator activities. Abscissa: concentration of activator in per cent of the stock solutions. Ordinate: increase in optical density at 275 mμ. (20 min., 37°, pH 7·6, casein method).

the assay. Further, the standard reference curve should be a correct expression of the relation between $E_0 - E_{20}$ and activator concentration.

RESULTS

Formation and properties of the activator of plasminogen in human blood

(i) *Formation and deterioration of activator at different concentrations of streptokinase.* By presupposing an enzymic effect of streptokinase on the proactivator, it should be possible to obtain a maximum formation of activator at low streptokinase concentrations, by increasing the reaction time at conditions under which the different components were stable. Preliminary experiments showed that the deterioration of activator was high at 37°, moderate at 22° and slow at 5°, and

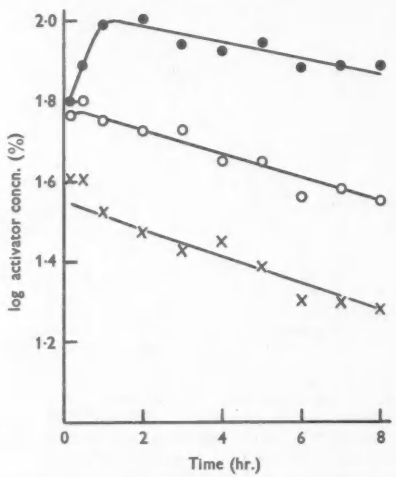


Fig. 4. The formation and deterioration of activator with time (pH 7·6, 22°) in solutions containing: human globulin (0·40 ml./ml.); ●, 8·0 mg.; ○, 1·6 mg.; ×, 0·8 mg. of streptokinase/ml. The activator concentrations were calculated as a per cent of the maximum concentration.

proactivator and streptokinase, kept separately, did not deteriorate significantly in 6 hr. at 22°. The formation and deterioration of activator at 22° were followed in solutions of human globulin containing an excess and suboptimum amounts of streptokinase (Fig. 4). After an initial increase, a slow decrease in activator concentration was observed in all instances. At the two lower streptokinase concentrations only a partial conversion of proactivator occurred. The logarithms of the activator concentration decreased proportionately to the reaction time, indicating that the loss of the activator was due to a simple protein denaturation, proceeding as a first-order reaction. The results contradicted a catalytic effect of streptokinase if activatable proactivator remained in the solutions containing suboptimum amounts of streptokinase. This assumption was verified by the increase in activator concentration produced by addition of more streptokinase to a solution of human globulin containing a small amount of streptokinase (Fig. 5). The results indicated a stoicheiometric reaction between streptokinase and proactivator.

The possibility remained that streptokinase might be bound progressively by antistreptokinase present in the globulin, whereby a catalytic process might appear as a stoicheiometric reaction. Experiments showed, however, that identical amounts of activator were formed at a suboptimum concentration of streptokinase (0·8 mg./ml.) in solutions containing 0·40 and 0·80 ml. of human globulin per ml., and thus significant amounts of antistreptokinase were probably not present in the globulin solution.

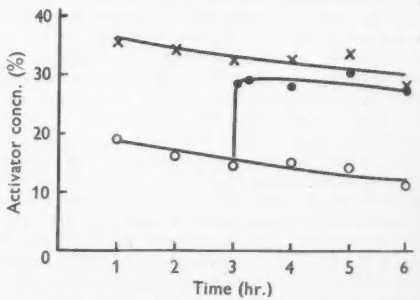


Fig. 5. Increase in activator concentration on the addition of streptokinase to a solution of human globulin containing a suboptimum amount of streptokinase. The solution contained human globulin (0·40 ml./ml.) and the following concentrations of streptokinase: ×, 1·6 mg./ml. for 6 hr.; ○, 0·8 mg./ml. for 6 hr.; ●, 0·8 mg./ml. for the first 3 hr. and 1·6 mg./ml. for the last 3 hr. (22°, pH 7·6, casein method).

(ii) *Mode of reaction between streptokinase and proactivator.* A study was made of the amount of activator formed at different proportions of streptokinase and proactivator. Two experiments were performed. In the first the globulin concentration was kept constant and the streptokinase concentration was varied; in the second the opposite was done. The amount of activator formed was expressed in arbitrary activator units, designating the maximum amount formed as 100 units. In the presence of an excess of one component, the

amount of activator formed was found to be proportionate to the concentration of the other component. It was therefore possible to express the concentration of streptokinase and globulin in activator units calculated from the number of units formed by one component in the presence of an excess of the other component. In the first experiment (streptokinase varied, globulin kept constant) a concentration of 0·60 mg. (98 units) of streptokinase/ml. was found to be equivalent to the concentration of the globulin used in all solutions (0·15 ml./ml. = 98 units/ml.). In the second experiment this streptokinase concentration was used in all solutions and the globulin concentration was varied above and below the calculated equivalent concentration. By plotting the amount of activator formed against the concentration of the varied component, the results of the two experiments conformed to a common curve (Fig. 6). In two

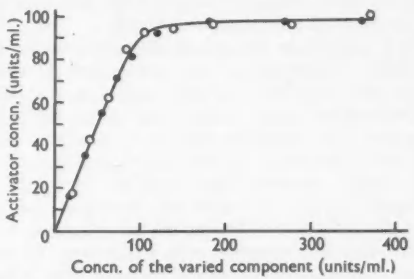


Fig. 6. Relation between the concentration of activator and of streptokinase in solutions containing a concentration of proactivator of 98 units/ml. (=0·15 ml. of human globulin/ml.), ●; and between the concentration of activator and of proactivator in solutions containing a concentration of streptokinase of 98 units/ml. (=0·60 mg./ml.), ○. The solutions were left for 2 hr. at 22° and pH 7·6 to complete the reaction before the determination of activator was performed by the casein method (pH 7·6, 37°).

similar experiments, with a different stock solution of streptokinase and about twice as concentrated solutions, 0·27 ml. of human globulin/ml. was found to be equivalent to 1·35 mg. of streptokinase/ml., and a curve of a similar shape was obtained. The experiments indicated a reaction proceeding to equilibrium (see Discussion).

On the assumption that two components, streptokinase and proactivator, combine in a reversible reaction to form a single component, activator, the concentration of activator in any mixture in equilibrium should be reduced by dilution. If two components, streptokinase and proactivator, react to form two components, activator and an inactive component, the equilibrium should not be influenced by dilution. The limit of accuracy of the estimations did not permit a consideration of more complicated reactions. The effect of dilution was studied in two different ways. In the first part of the experiment streptokinase was added to proactivator in equivalent concentrations and the reaction was allowed to proceed to equilibrium in a concentrated solution and in a solution diluted 1:10. In the second part of the experiment the concentrated solution containing the components in equilibrium was diluted 1:10 and kept for sufficient time to allow any changes in the equilibrium to take place. The activator contents of the concentrated and the two diluted solutions were then determined. The reduction in concentration of the reacting components due to the dilution was corrected for by performing the determinations on the diluted solutions by means of samples ten times larger than those from the concentrated solution. Identical final concentrations of the reacting components in the assay mixture were thereby obtained, and identical amounts of activator should be found in all instances, if the equilibrium had not been affected (Table 1). The equilibrium was not influenced by

Table 1. *Effect of dilution on the activator content of solutions containing equivalent amounts of streptokinase and proactivator*

A solution (*A*) containing human globulin (0·10 ml./ml.) and streptokinase (0·40 mg./ml.) was prepared. Samples were withdrawn and diluted 1:10 at once (*B*) and after 145 min. (*C*). After 265 min. the amounts of activator in samples of 25 μl. from *A* and of 250 μl. from *B* and *C* were determined and expressed as a per cent of the amount present in *A* (mean = 100%). Incubation: 22°, pH 7·6; determinations: casein method.

	A	*B*	*C*
Mean ± S.E.	100·0 ± 0·51	97·9 ± 0·49	100·4 ± 0·63
No. of results	(10)	(10)	(10)
Range	98·0–102·3	94·4–99·8	99·3–105·2

diluting a solution containing the components in equilibrium, but slightly smaller amounts of activator were formed in a diluted than in a concentrated solution. In control experiments the concentration of activator was determined at short intervals of time in the concentrated and the two diluted solutions. A somewhat lower rate of reaction between streptokinase and proactivator in the diluted solution was observed, but the difference was too small to effect a significantly greater loss of activator in the concentrated than the diluted solution during the experiment. No significantly different stability was observed.

(iii) *Stability of the plasminogen activator.* A solution containing human globulin (0·40 ml./ml.) and streptokinase (8 mg./ml.) was left at 22° for 3 hr., to obtain a complete conversion of pro-activator into activator, and then diluted 1:40. Samples were adjusted to different pH values, in an ice bath, with 0·15N-NaOH or 0·15N-HCl and 1/16 volume of an appropriate buffer, diluted to equal volumes, heated at 22°, 37° and 50° for 30 min., cooled, and brought back to pH 7·6 by addition of 0·15N-NaOH or 0·15N-HCl and phosphate buffer and readjusted to equal volumes. The residual amount of activator was estimated by the casein method. The results (Fig. 7) showed the activator to be very labile below pH 5 or above pH 9–10 with an optimum stability between pH 6·5 and 8·0. Identical results were obtained with a different preparation of activator (casein method) and with concentrated and diluted solutions of another activator preparation (standard fibrin plate method). Activator was also found to be destroyed at pH 2·9 in the presence of its substrate, plasminogen. Solutions of bovine plasminogen and activator were mixed and the pH was immediately brought to pH 2·9. After neutralization, only insignificant amounts of plasmin were formed in this mixture, as compared with control solutions (Table 2). The stability of the activator differed considerably from that of human plasminogen (Christensen & Smith, 1950; Kline, 1953), streptokinase (Christensen, 1947*b*) and human plasmin (see below), especially at acid reactions. This difference in stability made it possible to prepare human plasmin free from activator, and shows that the effect on bovine plasminogen of solutions of human globulin and streptokinase is caused by a substance different from previously known components of the proteolytic-enzyme system of blood.

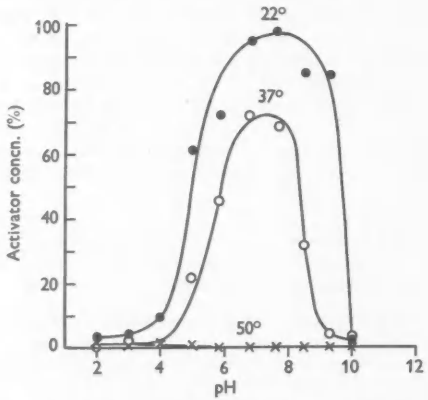


Fig. 7. Residual concentration of activator in solutions heated at different pH values and temperatures for 30 min. The concentration of activator is expressed as per cent of that of an untreated sample kept at pH 7·6 and 0° during the experiment (casein method). The values were corrected for the effect of the plasmin contained in the samples, as determined on casein without added plasminogen.

(iv) *Activation of bovine plasminogen by the activator.* Experiments were performed to determine the conditions under which a complete activation of bovine plasminogen by the activator occurred. The smallest amount of activator that produced maximum activation of plasminogen in 1 hr. at 22° was established and the formation of plasmin at this activator concentration, pH 7·4 and 22°, was followed. In the incompletely activated samples removed within the first 60 min. more plasmin was formed during the assay, resulting in concave digestion curves, and the plasmin activity, *k*, was determined by means of tangents drawn to the initial part of these curves. No further activation of plasminogen during the assay occurred in the samples removed subsequently. The activation curve (Fig. 8) indicated a complete initial activation, followed by a slow deterioration of the plasmin formed. Addition of an excess of activator to the mixture after activation for 4 hr. did not increase the activity significantly.

Table 2. *Deterioration of activator at acid reaction in the presence of plasminogen*

Plasminogen: bovine, 10 mg./ml.; activator: human, 2% (v/v); buffer: phosphate buffer, pH 7·4; the activation mixtures contained 0·25 ml. of each component. Each mixture was finally diluted to 2·5 ml., added to an equal volume of casein and the plasmin activity ($E_0 - E_{20}$) was determined.

Activation mixture	Procedure (1)	$E_0 - E_{20}$	Procedure (2)	$E_0 - E_{20}$
1. Plasminogen + activator	Heated at pH 2·9, 22° for 30 min.; adjusted to pH 7·4 and added to casein	0·008	Heated at pH 2·9, 22° for 30 min.; adjusted to pH 7·4; kept at 22° for 30 min. and added to casein	0·033
2. Plasminogen + buffer		0·002		0·000
3. Activator + buffer		0·005		0·010
4. Plasminogen + activator	Kept at pH 7·4, 22° for 30 min. and added to casein	0·349	Kept at pH 7·4, 22° for 60 min. and added to casein	0·385

The formation of plasmin at different activator concentrations was studied in an experiment at 22° (4 hr.) and at 5° (48 hr.). In all instances a progressive activation of plasminogen at small activator concentrations and a slow deterioration of plasmin resulted in approximately identical final plasmin activities. The results suggested that the activation of plasminogen by activator was a catalytic reaction, and the preliminary results (Müllertz & Lassen, 1953), which indicated a stoicheiometric reaction, were probably caused by the lability of plasmin under the applied conditions (37°). However, the deterioration of plasmin and the continuous formation of plasmin during the assay make it difficult to obtain conclusive evidence as to the mode of reaction by these methods.

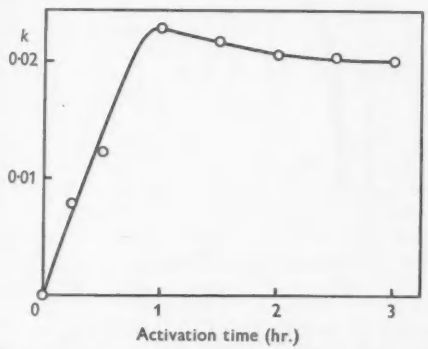


Fig. 8. Formation and deterioration of plasmin in a solution of bovine plasminogen (5 mg./ml.) containing 1% (v/v) activator (22°, pH 7·4). Samples were removed and diluted 1:5 with phosphate buffer and the plasmin activity was determined by the casein method (37°, pH 7·6). Control: activator + casein: 0·0001.

Properties of human and bovine plasmin

(i) pH *of optimum activity.* Samples of a 6% (w/v) casein solution were adjusted to different pH values with 0·15N-HCl and 0·15N-NaOH and diluted with appropriate buffer solutions to a final concentration of 3% (w/v). Casein and plasmin solutions were left at 37° until temperature equilibrium was reached (5 min.), and then equal volumes were mixed. The pH was controlled before and after the experiment by a glass electrode. Direct relationships between digestion time and increase in optical density were observed in all cases, indicating that no significant deterioration of plasmin occurred during the experiment. The pH activity curves for human and bovine plasmin were almost identical (Fig. 9), with a maximum of activity between pH 7·5 and 8·3 and a rapid decrease in activity above and below this range. Similar results were obtained with a bovine plasmin activated by chloroform as estimated on gelatin (Kaplan, Tagnon, Davidson & Taylor, 1942) and with human plasmin activated by streptokinase or chloroform as estimated on casein (Ratnoff, 1948; Remmert & Cohen, 1949) or gelatin (Christensen & MacLeod, 1945).

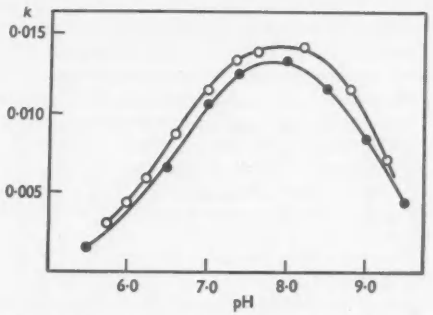


Fig. 9. pH of optimum activity of: ○, bovine plasmin; ●, human plasmin (casein method, 37°).

(ii) *Stability.* Samples of human and bovine plasmin were placed in an ice bath and adjusted to different pH values with 0·15N-HCl and 0·15N-NaOH and 0·1 vol. of an appropriate buffer. The samples were heated at different temperatures for 30 min., cooled, brought back to pH 7·6 and diluted with phosphate buffer (pH 7·6) to twice the original volume. The pH was controlled before and after heating by a glass electrode. The residual activity was determined by the casein and the heated fibrin plate method. The results of the casein experiments (Figs. 10, 11) were confirmed by the fibrin plate experiments. The high stability of human plasmin at pH 1·8 and 100° was established in experiments with three different plasmin preparations and three crude samples of globulin activated by streptokinase, with both casein and heated fibrin as substrates. The high stability of bovine plasmin at pH 3 was confirmed in experiments with the same plasmin, and a plasmin prepared from another batch of plasminogen (casein method). At pH 6·0–8·0 human plasmin was fairly stable, but bovine plasmin was very labile. Human and bovine plasmin were completely stable for 20 min. at 37° in the presence of casein (Fig. 1).

(iii) *Effect of human plasmin on bovine and human plasminogen.* The formation of plasmin in serum or globulin solutions during storage or after chloroform treatment has been ascribed to an autocatalytic reaction (Christensen, 1947*a*; Rocha e Silva & Rimington, 1948; Kocholaty, Ellis & Jensen, 1952) and the effect of streptokinase and human globulin

on bovine plasminogen might be explained by a catalytic effect of human plasmin on bovine plasminogen. The plasminogen activator, present in high concentrations in the mixture of streptokinase and human globulin, was destroyed by the treatment at pH 1·8 and 100° for 30 min. used in the preparation of human plasmin. This plasmin produced no activation of bovine plasminogen, but human plasminogen activator activated the plasminogen in the presence as well as in the absence of human plasmin (Table 3). Similar results were obtained with human plasmin and plasminogen. No activation of bovine plasminogen was produced by a bovine plasmin activated spontaneously (Astrup, 1951) or by tissue activator (Astrup & Sterndorff, 1952).

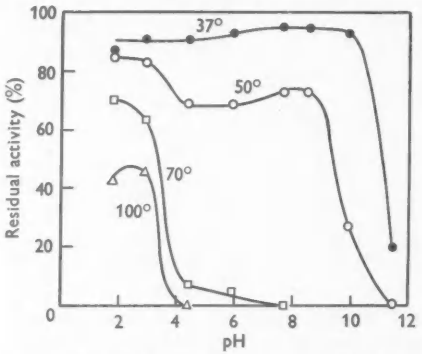


Fig. 10. Stability of human plasmin heated at different temp. and pH for 30 min. The residual activity (*k*, casein, 37°, pH 7·6) was expressed as a per cent of the plasmin content of a control kept at 0° and pH 7·6 during the experiment.

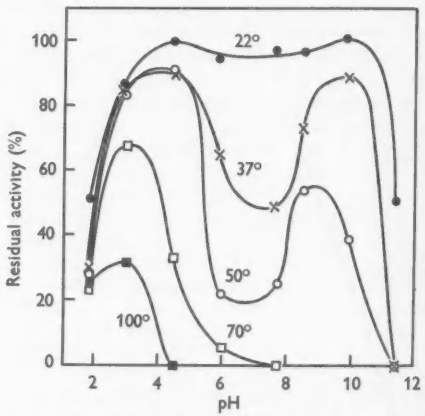


Fig. 11. Stability of bovine plasmin (see Fig. 10).

DISCUSSION

The experiments indicate that streptokinase and a component in human blood react to form an activator of plasminogen by a reaction proceeding to an equilibrium. The following two possible reactions will be considered:

(1) streptokinase + proactivator ⇌ activator,

(2) streptokinase + proactivator ⇌ activator + inactive component.

At the equilibria we have:

$$\frac{[A]}{([SK]-[A])\,([PA]-[A])} = K_1 \qquad (1)$$

and

$$\frac{[A]\,[B]}{([SK]-[A])\,([PA]-[A])} = K_2, \qquad (2)$$

where [SK] and [PA] represent the concentrations of streptokinase and proactivator added, [A] the activator concentration, [SK]−[A] and [PA]−[A] the free concentrations of streptokinase and proactivator respectively in the solution at equilibrium, and [B] the concentration of the inactive component. In the experiments considered here [B]=[A]. All concentrations are calculated in activator units/ml. In Fig. 4 the relation between [A] and [SK] (for [PA]=98) and between [A] and [PA] (for [SK]=98) was recorded. The points

Table 3. *Effect of human plasmin on bovine plasminogen*

Human plasmin: diluted 1:2 (Expt. 1) and 1:10 (Expt. 2), 1·0 ml.; bovine plasminogen: 2·5 mg./ml., 1·0 ml.; activator: human activator, 1% (v/v) 0·5 ml. All mixtures were diluted to 2·5 ml. with phosphate buffer (pH 7·4), kept at 22° for 60 min. and the plasmin activity ($E_0 - E_{20}$) was determined (casein). Controls of (1) bovine plasminogen and (2) activator yielded activities below 0·002.

Activation mixtures	$E_0 - E_{20}$ Expt. 1	$E_0 - E_{20}$ Expt. 2
1. Human plasmin	0·128	0·032
2. Human plasmin + bovine plasminogen	0·103	0·015
3. Human plasmin + bovine plasminogen + activator	0·429	0·382
4. Human plasmin + activator	0·106	0·029
5. Bovine plasminogen + activator	0·350	0·350

represent the experimentally determined values, and the curve is calculated from eqn. 1 for $K_1 = 1$. The curve is almost coincident with another curve calculated from eqn. 2 for $K_2 = 87$, and the accuracy of the determinations did not allow further conclusions about the mode of reaction. For equivalent concentrations of streptokinase and proactivator we have [SK] = [PA] = [C] and according to (1):

$$\frac{[A]}{([C]-[A])\,([C]-[A])} = K_1,$$

or

$$[C] = [A] + \sqrt{\left(\frac{[A]}{K_1}\right)}.$$

By inserting the highest value of K_1 that will fit the experimental data of Fig. 4 ($K_1 = 2$) the minimum theoretical effect of dilution on the equilibrium (1) could be calculated. Tenfold dilution should reduce the concentration of activator to 8·2 % ($K_1 = 1$) or 8·6 % ($K_1 = 2$) of the concentration in the undiluted solution. The activator concentration of a solution in equilibrium was not reduced below the 10 % that would be expected if equilibrium (2) were valid. However, a slight reduction to 9·8 % was observed if streptokinase and globulin were mixed and allowed to react completely in undiluted and diluted solution. The latter observation cannot be explained from the evidence available here. Nevertheless the results do not agree with a reaction such as (1), but suggest that equilibrium (2) is valid.

Previous studies have led to different concepts of the mechanism of formation of human plasmin by streptokinase. Christensen & MacLeod (1945) found that the transformation of human plasminogen into plasmin was a first-order reaction whose rate was proportional to the concentration of streptokinase within certain limits. They concluded that the reaction was catalysed by streptokinase. Remmert & Cohen (1949) confirmed this observation. Ratnoff (1948) observed a catalytic effect of streptokinase on a heated human plasminogen, and streptokinase appeared to react stoicheiometrically with untreated plasminogen. Wasserman (1952) found that the reaction between streptokinase and human plasminogen was stoicheiometric, when assayed by means of a substrate of clotted bovine fibrinogen. However, his results obtained with this plasminogen-containing substrate are probably pertinent to the formation of activator by the interaction of streptokinase and proactivator, whereas the formation of plasmin was followed by Christensen & MacLeod by means of gelatin, and by Remmert & Cohen and Ratnoff by means of casein. In the present paper, streptokinase and proactivator were found to interact stoicheiometrically and it was found most probable that the activator catalysed the transformation of plasminogen into plasmin. If these concepts are correct, the formation of plasmin on the addition of streptokinase to preparations containing both proactivator and plasminogen will appear as a catalysed reaction.

The presence of large amounts of plasminogen-activating substances in different fibrinolytic preparations from human blood has not been previously realized, and may explain a number of discrepancies in previous studies. The high lytic activity produced in a bovine-fibrin substrate by human globulin, activated by streptokinase, is due primarily to activation of the plasminogen in the substrate (cf. Müllertz & Lassen, 1953; Sherry, 1954). Previous studies on the interaction of streptokinase, human plasminogen and plasmin, performed by means of a bovine-fibrin substrate, are therefore primarily pertinent to the proactivator–activator system and only to a very slight degree to plasminogen and plasmin. This also applies to fibrinolytic methods generally used for assay (Astrup & Müllertz, 1952; Christensen, 1949; Fletcher, 1954). Discrepancies observed in studies on fibrinolysins occurring in blood in living and dead organisms (for references see Bidwell, 1953) are probably also caused by activation of plasminogen in the fibrin substrates by an activator in the lytic samples (Müllertz, 1953).

Reports on the stability of plasmin have not been concordant. In the present paper completely activated preparations of human and bovine plasmin free from inhibitory and activating components have been made. Changes in activity of incompletely activated enzyme preparations which contain activating substances and inhibitors may depend on destruction or enhancement of these factors and cannot be evaluated with certainty. Loss of activity during the preparation has therefore been considered of minor importance. In the present work, human and bovine plasmin showed a remarkable high stability at acid reaction. Sherry (1954) also noted a high stability of human plasmin at pH 2. Christensen & MacLeod (1945) found a maximum stability of human plasmin at pH 7·0–7·4, with a rapid loss above and below these values in the pH range 3·5–11. An incomplete activation or loss of plasmin by adsorption on protein denatured by heating at acid reaction and removed before the assay by viscosimetry on gelatin may be considered as an explanation of this discrepancy. At neutral reaction human plasmin was found to be rather labile, the stability being increased considerably by heating and chloroform treatment (Ratnoff, 1948) or by addition of casein or esterified basic amino acids (Kline, 1954). It was also found by Christensen (1945) to be fairly stable. Bovine plasmin was found to be very labile at neutral reaction and the stability curve in the pH range 5·0–9·5 was approximately the inverse of

the pH/activity curve. Bovine plasmin was stabilized in the presence of casein. Lewis & Ferguson (1951) found a similar stability of a dog-blood lysin in the pH range 5·0–9·7. The results indicate that both human and bovine plasmin are autodigestive. Thus the rate of autodigestion should be high in purified preparations, whereas a competitive inhibition occurs in crude preparations in the presence of added protein or after heat or chloroform treatment, which make contaminating serum globulins more susceptible to plasmin.

SUMMARY

1. An activator of plasminogen is formed by the interaction of streptokinase and a component, a proactivator, in human blood.

2. At suboptimum concentrations of streptokinase, only a partial conversion of proactivator into activator was obtained, followed by a slow deterioration of the activator formed. The amount of activator formed in solutions containing varying proportions of streptokinase and of proactivator indicated a stoicheiometric reaction proceeding to an equilibrium. Experiments on the effect of dilution suggested that streptokinase and proactivator interact to form two components, an activator and an inactive component.

3. The activator was very labile at acid and alkaline reactions, with an optimum of stability between pH 6·5 and 8·0, differing in this respect from other known components of the proteolytic–enzyme system in blood.

4. A complete conversion of bovine plasminogen into plasmin was accomplished by addition of the activator. The preparations of human and bovine plasmin were purified by a treatment at acid reaction, which produced a destruction of streptokinase, activator and inhibitory substances.

5. Human plasmin did not catalyse the conversion of human and bovine plasminogen into plasmin. Human and bovine plasmin showed nearly identical relationships between pH and activity on casein, with an optimum between pH 7·5 and 8·3. They showed a remarkably high stability at acid reaction. At neutral reaction human plasmin was fairly stable, while bovine plasmin was rather labile. Plasmin was stabilized by casein.

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Phosphate-Transfer Reactions of Adenosine and Inosine Nucleotides

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It was shown in a previous paper (Krebs & Hems, 1953) that animal tissues contain an enzyme catalysing a rapid transfer of phosphate between adenosine- and inosine-di- and -tri-phosphates according to the scheme

$$ATP + IDP \rightleftharpoons ITP + ADP, \quad (1)$$

where ATP and ADP stand for adenosine tri- and di-phosphates, and ITP and IDP for inosine tri- and di-phosphates respectively. Berg & Joklik (1953, 1954) found this reaction simultaneously and independently in yeast. The present paper is concerned with another phosphate-transfer reaction of animal tissues:

$$ITP + AMP \rightleftharpoons ADP + IDP, \quad (2)$$

where AMP stands for adenosine monophosphate. While the experiments establish the occurrence of reaction (2), they leave the mechanism of the phosphate transfer undecided. Reaction (2) is analogous to that catalysed by adenylate kinase (myokinase).

$$ATP + AMP \rightleftharpoons 2\ ADP, \quad (3)$$

but purified adenylate kinase of muscle tissue does not catalyse reaction (2). It is possible that the enzyme is identical with the adenylate kinase of other tissues, which, according to Kotelnikova (1949, 1950) and Barkulis & Lehninger (1951), differs from the muscle-adenylate kinase. It is also possible that the transfer is the result of the combined action of reactions (3) and (1). In this case catalytic quantities of ADP and ATP would participate in the reaction. No detectable quantities of these reagents were present at the beginning of the incubation period, but this does not rule out the presence of catalytic amounts.

MATERIALS AND METHODS

ATP was prepared according to LePage (1949) and ITP according to Kleinzeller (1942). ADP was obtained from Schwarz Laboratories Inc., New York, and AMP from Roche Products Ltd., Welwyn Garden City; IDP was prepared by deamination of ADP with nitrous acid, the directions given by Kleinzeller (1942) for the deamination of ATP being followed. ATP and ITP contained ADP and IDP respectively. The quantities of these impurities present at zero time are stated in the tables.

ATP containing ^{32}P in the β- and γ-positions was prepared according to Hems & Bartley (1953). β-Labelled ADP was a by-product in the preparation of labelled ATP and was separated chromatographically. γ-Labelled ITP was prepared by the procedure of Hems & Bartley, by including ITP as well as ATP among the added reagents and separating the products by paper chromatography. About 92% of the isotope was in the γ-phosphate and about 8% in the β-phosphate of ITP. The sample also contained some β-labelled IDP. Labelled AMP was prepared according to Eggleston (1954). The specific activity of the nucleotide phosphorus was of the order of 10^6 counts/min./μg. The substances could therefore be diluted about 1000-fold with carrier material.

Sheep-liver mitochondria were prepared by homogenizing fresh liver with 3 vol. of 0·25 M-sucrose in the apparatus of Potter & Elvehjem (1936) made of stainless steel. The supernatant (30 ml.) obtained on centrifuging at 150 *g* for 15 min. was mixed with 1·5 M-KCl (4 ml.) and this was centrifuged for 15 min. at 2500 *g*. The sediment of mitochondria was resuspended in 0·5% KCl and centrifuged at 2500 *g* for 7 min. This suspension was again centrifuged and the residue was suspended in 5 vol. of water. The suspension was frozen at about $-15°$. After thawing, the material was centrifuged at 24000 *g* and the supernatant was used as enzyme preparation. It was stored at $-15°$.

Acetone-dried powder of sheep brain was obtained by freezing brain tissue in solid CO_2 as soon as possible after the death of the animal. The solid material was broken up into smaller pieces in a cooled mortar and then disintegrated in a Waring Blendor with 10 vol. of acetone at $-15°$. The insoluble material was filtered off and washed three times with 10 vol. of cold acetone. The powder was finally dried in a desiccator over H_2SO_4 and stored *in vacuo* in the cold. Aqueous extracts of the powder were made by homogenizing it with 20 vol. of water and centrifuging it in the cold at 20000 *g*. The pink, almost clear supernatant served as enzyme solution.

RESULTS

Interaction between AMP and ITP in lysed liver mitochondria

The reaction mixture contained 10% (v/v) of the lysed mitochondria preparation, 0·003 M-$CaCl_2$, 0·035 M-$NaHCO_3$ and initially the amounts of AMP, ITP and IDP stated in Table 1. The ITP sample contained ^{32}P in the terminal phosphate group, and small amounts also in the β-phosphate. Three parallel mixtures were set up, the first containing both AMP and ITP, with some IDP, the

Table 1. *Reaction between AMP and ITP (labelled with* ^{32}P *in terminal* P) *in sheep-liver mitochondria*

For conditions see text.

Substance to which results refer	AMP, ITP (and IDP) added						ITP (and IDP) added			AMP added		
	P found (μg./25 μl.)		Change (mμmoles)	Counts/min./25 μl.		Specific activity (counts/min./μg. P)	P found (μg./25 μl.)		Change (mμmoles)	P found (μg./25 μl.)		Change (mμmoles)
	0 min.	60 min.		0 min.	60 min.	0 min.	0 min.	60 min.		0 min.	60 min.	
ITP	4·78	2·27	−27·0	8548	3901	1788	4·71	4·03	−7·3	—	~0	—
IDP	2·17	1·29	−14·2	649	356	299	2·21	1·22	−16·0	—	~0	—
IMP	0	1·38	+44·5	0	205	0	148	0	0·91	+29·3	—	—	~0	—
ATP	0	0·21	+2·3	0	670	0	3190	—	~0	—	—	~0	—
ADP	0	1·05	+17·0	0	2563	0	2440	—	~0	—	—	~0	—
AMP	3·83	3·13	−22·6	3	68	0	21	—	~0	—	3·81	3·49	−10·3
Inorganic P	0·36	1·74	+44·5	159	1375	440	790	0·49	1·48	+31·9	0·06	0·22	+5·2

second ITP with some IDP, and the third AMP. The samples were saturated with CO_2+N_2 (5:95) at 30°. For analysis a 0·5 ml. sample was mixed with 0·05 ml. of 30 % (w/v) trichloroacetic acid and 25 μl. were chromatographed according to Krebs & Hems (1953).

As will be seen in Table 1, the initial quantities of AMP, ITP and IDP present in the first mixture decreased on incubation and ATP, ADP and IMP (inosine monophosphate) were formed. Two types of reaction can be visualized as causing a formation of ATP and ADP. Either an amino group is transferred from AMP to the inosine phosphates (transamination) or phosphate is transferred from inosine phosphates to AMP (transphosphorylation). In transamination the specific activities of ATP and ITP and ADP and IDP would be the same. In fact the specific activity of ATP was about twice that of ITP and that of ADP a multiple of that of IDP. These findings disprove the occurrence of transamination, but are in accordance with the assumption that transphosphorylation occurs, the radioactive phosphate of ITP providing the β- and γ-phosphates of ATP and the β-phosphate of ADP. The quantitative changes agree with the assumption that the reactions listed in Table 2 take place. The procedure for obtaining the data in Table 2 was as follows. As ATP participates in reaction *b* only, the amounts reacting according to *b* are equal to the amount of ATP formed. ADP participates in

Table 2. *Reactions accounting for the changes on addition of AMP, ITP and IDP recorded in Table* 1

Reactions	Quantities reacting in 60 min./25 μl. of soln. (mμmoles)
a ITP + AMP = IDP + ADP	20
b ITP + ADP = ATP + IDP	2·3
c ITP = IDP + P	4·7
d IDP = IMP + P	41·3
e AMP = IMP + NH_3	3

Table 3. *Comparison of changes observed (see Table* 1), *and calculated from Table* 2

The values refer to 25 μl. of solution and 60 min.; AMP, ITP and IDP added.

Substances	Changes	
	Calc. (mμmoles)	Obs. (mμmoles)
ITP	−27·0	−27·0
IDP	−14·5	−14·2
IMP	+44·3	+44·5
ATP	+2·3	+2·3
ADP	+17·7	+17·0
AMP	−23·0	−22·6
Inorganic P	+46·0	+44·5

a and *b* only and so, if *b* is known, the amount of *a* can be calculated from the amount of ADP formed. Similarly, *c*, *d* and *e* can be calculated. Since the number of values obtained experimentally is greater than the number of quantities to be solved for, there is a choice of procedures for calculating *a*, *b*, etc.; and since the values are subject to experimental error, the values obtained will differ according to the procedure chosen. Accordingly one procedure was chosen arbitrarily to give a set of values of *a*, *b*, etc., as a first approximation, and these values were then finally adjusted to give the best general agreement between the observed and predicted changes in the quantities of the various substances (Table 3). A statistical method of solution might have been used, but would not have been profitable since the weights to be attached to the experimental values were not known.

The agreement between the expected and observed changes shown in Table 3 is satisfactory and testifies to the reliability of the analytical methods. A further check on the reliability of the results is afforded by the balance of the changes as shown in Table 4. The agreement is again satisfactory. As expected from Table 2 there is a small loss (3·3 μmoles) of adenosine nucleotide and an equivalent gain of inosine nucleotide.

The only discrepancy concerns inorganic phosphate, where the observed value is too low by 229 counts. This may be due to imperfect separation of the phosphates and in particular to contamination of IMP with inorganic phosphate. Most of the missing counts (205) were found in the IMP area, though it is unlikely that IMP itself was radioactive.

The changes observed in the control containing ITP, with some IDP, as recorded in Table 1, are in agreement with the assumption that both substrates undergo dephosphorylation. The quantitative aspects are recorded in Tables 6 and 7. There is a discrepancy between the IMP value calculated and observed, but this is within the limits of error of the analytical methods ($\pm 4\%$). The change of ITP is probably not very accurate as it is a relatively small difference value. The rate of dephosphorylation of ITP is much slower than that of IDP.

The changes in the control containing only AMP do not balance satisfactorily, in that the AMP removed is not accounted for by the substances which were determined. Other substances such as adenosine or inosine must have been formed. Dresel (1953) has demonstrated the instability of nucleotides in liver preparations.

Table 4. *Balance of changes on incubation (experiment of Table 1)*

The values refer to 25 μl. of solution.

Total nucleotides ...	63·8 mμmoles formed; 63·8 mμmoles removed
Adenosine phosphates ...	19·3 mμmoles formed; 22·6 mμmoles removed
Inosine phosphates ...	44·5 mμmoles formed; 41·2 mμmoles removed
Total P	11·14 μg. at 0 min.; 11·07 μg. at 60 min.
Counts/min.	9359 at 0 min.; 9138 at 60 min.

Table 5. *Analysis of radioactive counts*

The calculated values are based on the assumption that the reactions listed in Table 2 took place, and refer to counts/min. in 25 μl. of solution.

Substance	Changes in counts: Calc.	Obs.
ADP	+2560	+2563
ATP	+ 666	+ 760
Inorganic P	+1604	+1375

The conclusions formulated in Table 2 are confirmed by the quantitative analysis of the distribution of radioactivity. The numbers of counts expected in ADP, ATP and phosphate have been calculated on the assumption that the reactions listed in Table 2 take place in the quantities stated in the last column, and that the specific activity of the β-phosphate of ITP was identical with that of IDP. The agreement between the calculated and observed counts is again very good (Table 5). The

Table 6. *Reactions accounting for changes on addition of ITP and IDP recorded in Table 1*

Reactions	Quantities reacting in 60 min./25 μl. of soln. (mμmoles)
ITP = IDP + P	7·3
IDP = IMP + P	23·3

Table 7. *Comparison of changes observed (see Table 1), and calculated from Table 6*

The values refer to 25 μl. of solution and 60 min.; ITP and IDP added.

Substance	Changes: Calc. (mμmoles)	Obs. (mμmoles)
ITP	− 7·3	− 7·3
IDP	−16·0	−16·0
IMP	+23·3	+29·3
Inorganic P	+30·6	+31·9

Interaction between AMP and ITP in brain extract

A similar experiment in which the liver preparation was replaced by an aqueous extract of acetone-dried sheep brain is shown in Table 8. The composition of the incubated solution was the same as in the previous experiment, except for the source of the enzyme. The results are in good agreement with the assumption that essentially the same five reactions are responsible for the changes in brain and in liver. The quantitative aspects are stated in Tables 9–12. They confirm the agreement between the various independent checks of the results. There are some differences in the relative activities of the enzymes in the brain and liver preparations. In liver the dephosphorylation of IDP was a more prominent reaction than in brain. The deamination of AMP was much more rapid in brain than in liver. It should be noted that the enzyme preparation did not comprise all the enzymes of the original tissue and the comments on the relative activities therefore refer only to the preparations used.

The distribution of ^{32}P shows some unexpected features which may be due to experimental errors, e.g. the fall of specific activity of IDP on incubation (Table 8). This is probably due to an error in the count for the IDP phosphorus at 0 min. As the same specimen was used as in the experiment of Table 1, the ratio of the counts of ATP and ADP should be the same in both experiments. Another doubtful value is the radioactivity count in IMP after incubation. The reactions listed in Table 2 do not account for this. Whether additional reactions take place or whether the chromatographic separation is imperfect remains to be investigated.

Table 8. *Reaction between AMP and ITP (labelled with ^{32}P in terminal P) in sheep-brain acetone powder extract*

For conditions see text.

Substance to which results refer	AMP and ITP (and IDP) added: P found (μg./25 μl.) 0 min.	P found (μg./25 μl.) 120 min.	Change (mμmoles)	Counts/min. 0 min.	Counts/min. 120 min.	Specific activity (counts/min./μg. P) 0 min.	Specific activity (counts/min./μg. P) 120 min.
ITP	5·36	2·17	−34·3	8795	3157	1640	1453
IDP	2·29	4·05	+28·4	946	817	413	202
IMP	0	1·58	+51·0	0	241	0	152
ATP	0	0·63	+ 6·8	0	1624	0	2580
ADP	0	1·32	+21·3	0	3032	0	2295
AMP	4·13	1·85	−73·3	13	71	0	38
Inorganic P	0·63	1·10	+11·9	465	800	738	726

Table 9. *Reactions accounting for the changes recorded in Table 8*

Reactions	Quantities reacting in 120 min./25 μl. of soln. (mμmoles)
ITP + AMP = IDP + ADP	28
ITP + ADP = ATP + IDP	6·7
ITP = IDP + P	1
IDP = IMP + P	7
AMP = IMP + NH_3	45

Table 10. *Comparison of changes observed (see Table 8) and changes calculated from Table 9*

The values refer to 25 μl. of solution and 120 min.; AMP, ITP and IDP added.

Substance	Changes: Obs. (mμmoles)	Changes: Expected (mμmoles)
ITP	−34·3	−35·7
IDP	+28·4	+28·7
IMP	+51·0	+52
ATP	+ 6·8	+ 6·7
ADP	+21·3	+21·3
AMP	−73·3	−73
Inorganic P	+11·9	+ 8

Table 11. *Balance of changes on incubation for the experiment of Table 8*

The values refer to 25 μl. of solution.

Total nucleotides ...	107·5 mμmoles formed; 107·6 mμmoles removed
Adenosine nucleotides ...	28·1 mμmoles formed; 73·3 mμmoles removed; loss, 45·2 mμmoles
Inosine nucleotides ...	79·4 mμmoles formed; 34·3 mμmoles removed; gain, 45·1 mμmoles
Total P	12·41 μg. at 0 min.; 12·70 μg. at 120 min.
Counts/min.	10 219 at 0 min.; 9742 at 120 min.

Reversibility of interaction between ITP and AMP

The reversibility of the reaction

$$ITP + AMP \rightarrow ADP + IDP$$

is shown by an experiment in which IDP and ADP were added to lysed liver mitochondria. The experimental conditions were the same as in the experiment shown in Table 1 except that the substrates added were ADP, labelled with ^{32}P in the terminal phosphate group, and IDP. Some IMP and AMP also were initially present as contaminations of the IDP and ADP preparations used; the initial quantities of IMP and AMP are stated in Table 13 under zero time.

On incubation the concentrations of IDP and ADP decreased and ATP, AMP, ITP and IMP were formed, as well as inorganic phosphate (Table 13). The balance of the changes of phosphate and nucleotides shows deficits which are above the limits of error and indicate that substances in addition to those estimated were formed, e.g. purines, ribosides, ribose phosphate and pyrophosphate. The results are roughly in agreement with the assumption that the reactions listed in Table 14 take place. It will be seen from Table 15 that some changes of IDP and IMP remain unaccounted for; the balance of the substances removed and recovered not being perfect (see Table 16), it is impossible to formulate the changes as satisfactorily as in the previous two experiments.

The radioactivity measurements qualitatively confirm the reactions formulated in Table 14. ^{32}P appeared in ITP, the gain being 516 counts. The expected gain was 897 counts. ADP lost 864 counts but the specific activity rose, which indicates the formation of ADP from a highly active ^{32}P donor. This was a trace of ATP present in the radioactive ADP in quantities which could

Table 12. *Analysis of radioactive counts for the experiment of Table* 8

The calculated values are based on the assumption that the reactions listed in Table 9 took place and that the specific activity for the β-P of IDP and ITP was 202 counts/min./μg. P. The values are for counts/min. in 25 μl. of solution.

Substance	Changes in counts: Calc.	Obs.
ADP	+2995	+3032
ATP	+1880	+1624
Inorganic phosphate	+ 784	+ 800

Table 13. *Reaction between IDP and ADP (labelled with ^{32}P in β-P) in sheep-liver mitochondria*

Substrates added: ADP, IDP and small amounts of IMP; 30°; for other conditions see text.

Substance to which results refer	P found (μg./25 μl.): 0 min.	60 min.	Change (mμmoles)	Counts/min.: 0 min.	60 min.	Specific activity (counts/min./μg. P): 0 min.	60 min.
ITP	~0	1·95	+21·0	39	555	—	265
IDP	8·47	5·74	−44·1	94	101	—	—
IMP	0·38	0·81	+13·8	53	80	—	—
ATP	<0·05	1·85	+19·9	1602	1524	—	822
ADP	6·41	2·69	−59·9	2551	1691	397	628
AMP	0·42	1·47	+33·9	10	6	—	—
Inorganic P	0·44	0·91	+15·1	392	613	890	675

Table 14. *Reactions accounting for the changes on addition of ADP and IDP recorded in Table* 13

Reactions	Quantities reacting in 60 min./25 μl. of soln. (mμmoles)
$ADP + IDP = ITP + AMP$	21
$2ADP = ATP + AMP$	40 and 20 respectively
$IDP = IMP + P$	15
$AMP = IMP + NH_3$	7

Table 15. *Comparison of changes observed (see Table* 13), *and calculated from Table* 14

The values refer to 25 μl. of solution and 60 min.; ADP and IDP added.

Substances	Changes: Calc. (mμmoles)	Obs. (mμmoles)
ITP	+21	+21·0
IDP	−36	−44·1
IMP	+22	+13·8
ATP	+20	+19·9
ADP	−61	−59·9
AMP	+34	+33·9
Inorganic P	+15	+15·1

Table 16. *Balance of changes on incubation for the experiment of Table* 13

The values refer to 25 μl. of solution.

Total nucleotides ...	88·6 mμmoles formed; 104·0 mμmoles removed
Adenosine phosphates ...	53·8 mμmoles formed; 59·9 mμmoles removed
Inosine phosphates ...	34·8 mμmoles formed; 44·1 mμmoles removed
Total P	16·17 μg. at 0 min.; 15·42 μg. at 60 min.
Counts/min.	4741 at 0 min.; 4570 at 60 min.

not be determined as phosphate, but which, owing to its high specific activity (10^5–10^6 counts/min./μg. P), added significantly to the radioactivity of the solution. If allowance is made for the conversion of ATP phosphorus into ADP phosphorus and the liberation of inorganic phosphate from ATP, the initial counts in ATP should have fallen from 1602 to 760. The number of counts found in ATP at the end of the incubation was greater by 764 counts. This is somewhat less than expected (996) but of the right order of magnitude.

Reaction between ATP and IMP

Brain extract was incubated with ATP (containing small amounts of ADP) and IMP under the conditions applying to Table 8. No interaction took place, although AMP and ITP readily interact under these conditions. ATP reacted in the presence of IMP in the same way as in its absence, i.e. a slow dephosphorylation occurred, and once AMP was formed the adenylate kinase reaction took place. No conditions in which IMP was converted into other nucleotides have been found. When it disappeared, it was by hydrolysis to inosine and possibly hypoxanthine.

Effects of inhibitors

The phosphate-transfer reactions described in this paper are, like those catalysed by ATPase and adenylate kinase, completely inhibited by 0·01 M ethylenediaminetetraacetate (EDTA). NaF (0·05 M) also causes inhibition. Additions of Mg or Ca ions are not necessary, but $CaCl_2$ (0·003 M) inhibits the liberation of inorganic phosphate from nucleotides. It was added because it stabilizes the preliminary products of phosphate transfer.

DISCUSSION

The results leave no doubt of the occurrence, in brain and liver extracts, of the reversible reaction:

$$ITP + AMP \rightleftharpoons ADP + IDP.$$

As the material may have contained trace quantities of ATP this reaction could have been the result of the adenylate kinase reaction:

$$ATP + AMP \rightleftharpoons 2ADP,$$

combined with the reaction previously demonstrated:

$$ITP + ADP \rightleftharpoons ATP + IDP.$$

There is no direct evidence in support of this mechanism. Addition of catalytic quantities of ATP failed to accelerate the reaction; a more direct transfer of phosphate from ITP to AMP is an alternative mechanism. It is noteworthy that ATP and IMP do not interact under conditions where ITP and AMP react.

The occurrence of analogous phosphate-transfer reaction has already been demonstrated in the intact animal by Potter, Herbert, Takagi, Siekevitz & Brumm (1954), who demonstrated the phosphorylation of uridine mono- and di-phosphate in the presence of ATP. They did not investigate phosphate-transfer reactions involving inosine phosphates.

The presence of radioactivity in the β-phosphate of IDP and ITP prepared from respiring heart-muscle preparations after incubation with inosine phosphates and radioactive inorganic phosphate cannot be explained on the basis of the known reactions. Formation of labelled IDP might be due to dephosphorylation of $\beta\gamma$-labelled ATP to ADP and deamination of the ADP by the enzyme described by Webster (1953) and by Deutsch & Nilsson (1953). However, in this case the specific activities of ADP and IDP should be equal, whereas in fact that of IDP was much lower. This suggests the formation of IDP by phosphorylation of IMP.

SUMMARY

1. Extracts of acetone-dried sheep brain and lysed mitochondria of sheep liver have been shown to bring about the phosphate-transfer reaction: ITP + AMP → ADP + IDP. The evidence rests on paper-chromatographic separation and quantitative determinations of the reactants and on the distribution of radioactive phosphate.

2. The reaction was found to be reversible in sheep-liver mitochondria.

3. The reaction is inhibited by ethylenediaminetetraacetate and sodium fluoride.

4. ATP and IMP did not interact under conditions where ITP and AMP interacted.

5. Apart from interacting as stated under (1), ITP and AMP enter other reactions. The observed

changes in the concentrations of nucleotides taking place on addition of ITP and AMP can be accounted for by the transphosphorylations, dephosphorylations and deamination listed in Table 2. The changes taking place on addition of ADP and IDP are approximately, but not fully, accounted for by the reactions listed in Table 14.

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The Effect of Insulin on the Anaerobic Metabolism of Rat Diaphragm

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The mechanism by which insulin increases the rate of glucose utilization by muscle has not yet been established. Cori put forward the hypothesis that insulin activates muscle hexokinase (Price, Cori & Colowick, 1945), and the work of Chaikoff and his collaborators (Chernick & Chaikoff, 1951) on glucose metabolism in liver supports this view; but the difficulty of demonstrating activation of the enzyme by insulin in tissue extracts has led to some loss of confidence in the theory. Much recent work (reviewed by Stadie, 1954) has been based on a hypothesis that insulin facilitates the transport of glucose across the cell membrane. This facilitation has sometimes been visualized as the removal of a 'permeability barrier' to glucose which is presumed to exist in the membrane. A third hypothesis, which has been suggested in various forms by Polis, Polis, Kerrigan & Jedeikin (1949), Russell (1951), Haugaard, Marsh & Stadie (1951), and others, is that insulin increases glucose utilization by making more adenosine triphosphate (ATP) available to hexokinase, possibly by increasing the efficiency of oxidative phosphorylation.

It is difficult to provide experimental evidence to confirm any one of these hypotheses, assuming that one of them is, in fact, correct. In all probability no one line of attack will provide decisive evidence, but investigation of the effectiveness of insulin in muscle kept anaerobic was thought likely to be profitable for the following reasons. If either the Cori hypothesis or the 'permeability' hypothesis is correct, exclusion of oxygen from the muscle should not itself affect insulin activity, so long as more than enough ATP remains in the tissue to saturate hexokinase. ATP is regenerated, in anaerobic conditions, by glycolysis and by transfer of phosphate from phosphocreatine, so that the ATP concentration might be expected to remain above saturation level for some time after exclusion of oxygen. If the hypothesis that insulin increases the efficiency of oxidative phosphorylation is correct, however, exclusion of oxygen from the system should severely impair or abolish insulin activity. For this type of work the *in vitro* incubation of the isolated rat diaphragm was regarded as very suitable, and this paper reports the results of experiments using this technique. The effects of other methods of reducing ATP synthesis have been included for comparison.

No conclusions about the mechanism of insulin action can be drawn if glucose utilization is impaired because the ATP concentration falls below saturation level early in the experiment. It was therefore important to be certain that ATP was not, in these experiments, the limiting factor in glucose uptake. Two methods of checking this point have been used in two independent laboratories, with almost identical results. The concentration of ATP in anaerobic diaphragm muscle has been estimated directly by Walaas & Walaas (1952), who were also interested in the relationship between insulin effectiveness and ATP levels in muscle. They found that the ATP concentration in the aqueous phase of the muscle remained above

10^{-3} M ($0{\cdot}4 \times 10^{-3}$ mole/g. of wet tissue) even after an hour's anaerobic incubation. The values for 30 min. incubation reported in this paper are in fact more than three times as large as this.

Another method of approach is to demonstrate that, in the conditions of these experiments, hexokinase activity in the muscle is still capable of being stimulated. This can be done by incubating the muscle with a higher-than-normal concentration of glucose. Both Ottaway (1950) and Walaas & Walaas (1952) found that the rate of glucose uptake by the muscle was increased when the glucose concentration was increased, in anaerobic just as in aerobic experiments. It has been inferred that the design of the experiments to be described does not invalidate any conclusions which can be drawn from them about the correctness of the mechanisms of insulin action that have been outlined.

METHODS

Preparation of the diaphragms. Albino rats fasted for 16–24 hr. were used. Each rat was anaesthetized with Nembutal and the diaphragm was excised just before death. The muscle was quickly washed and divided into two or four pieces, and each piece was placed in the appropriate medium in a Warburg flask which had already been swept out with N_2 or $N_2 + CO_2$. Hemidiaphragms were incubated in 2 ml. of medium, one of each pair in medium containing insulin. Quarter diaphragms were incubated in 0·5 ml. of medium in the side arms of Warburg flasks. The flasks were placed in a Warburg bath at 37° and gassed while shaking. In the experiments with phosphate-buffered medium the flasks were gassed with N_2 for 5 min.; in many of the experiments 0·3 ml. of 5% pyrogallol in KOH was placed in the centre well of each vessel immediately before attachment to the manometer. In the experiments with bicarbonate-buffered medium the flasks were gassed with $N_2 + CO_2$ (95:5) for 10 min. These latter experiments were performed as nearly as possible according to the directions of Cohen (1950), except for the amount of tissue placed in each flask and the concentration of insulin. Cohen used three or four hemidiaphragms, pooled from several rats, in each flask. This is not a satisfactory procedure, because of the difference in metabolic state of diaphragms stored for differing lengths of time, and in all the experiments reported here only one hemidiaphragm was used for each flask.

Incubation media. The phosphate medium was that described by Stadie & Zapp (1947). The bicarbonate medium was Krebs–Ringer solution. Glucose (2 mg./ml.) was added to both media.

The insulin, a crystalline preparation low in zinc, made by Burroughs Wellcome & Co., was dissolved in a small portion of medium with the aid of a drop of dilute HCl, and the solution diluted to a concentration of 0·2 unit/ml. Cohen (1950) used 1·5 units/ml.

Methods of analysis. Glycolysis in the bicarbonate medium was estimated by manometric measurement of CO_2 liberation after 20 min. had been allowed for equilibration. Readings were taken at 5 min. intervals.

At the end of the experiment the pieces of diaphragm were removed from their flasks, blotted, weighed and dropped into 30% (w/v) aqueous KOH for analysis of glycogen content by the method of Good, Cramer & Somogyi (1933). Portions (0·1 ml.) of the medium were deproteinized and analysed for glucose by Somogyi's (1945) modification of Nelson's method. Lactic acid was estimated by the method of Barker & Summerson (1941).

For the estimation of ATP, about 200 mg. of muscle were frozen by means of solid CO_2, and pulverized after transfer to a percussion mortar chilled with solid CO_2. The powder was placed in a weighed tube containing 5 ml. of ice-cold 10% (w/v) trichloroacetic acid, and the contents were homogenized in a Potter homogenizer. The insoluble material was centrifuged off and re-extracted with 5 ml. of 5% trichloroacetic acid. The ATP in the combined extracts was estimated by the method described by Umbreit, Burris & Stauffer (1949). In two estimations 1 μmole of potassium glucose 1-phosphate (about five times the expected content of ATP) was added to the extract. The estimated concentration of ATP in these extracts was not higher than in similar extracts without added glucose phosphate.

The bicarbonate remaining in the Krebs–Ringer medium at the end of the experiment was estimated in a van Slyke manometric apparatus.

Paper chromatography for the detection of keto acids was carried out on 1–1·5 ml. samples of the medium, according to the suggestions of Altmann, Crook & Datta (1951).

RESULTS

Effect of insulin on rat diaphragm incubated anaerobically in a bicarbonate buffer

The experimental conditions used in much of this work are those described by Cohen (1950), who reported that insulin stimulated anaerobic glycolysis in rat diaphragm *in vitro*, in the presence of glucose. Cohen's report was at variance with preliminary results which had been obtained here, and has not been substantiated by the present work.

In a small series of experiments (Table 1, Series A) an increase in rate of glycolysis of about 14% above the control was observed in the diaphragms incubated with insulin, i.e. almost the same as the percentage increase reported by Cohen (1950). The percentage increase was of the same magnitude in the 20–50 min. and the 50–90 min. periods, but it was not statistically significant. There was no parallel increase in the rate of glucose uptake of the muscle incubated with insulin.

In further experiments (Table 1, Series B and C, and Table 2) no increase in the glycolysis rate in the presence of insulin was consistently observed. It must be concluded that the increase in the preliminary experiments, and possibly that observed by Cohen, was due to chance. There was again no difference between the glucose uptake of diaphragm incubated with or without insulin. The results of the 20 min. incubations are, for technical reasons, not very reliable, but in them too there is no

Table 1. *Carbon dioxide output and glucose uptake by rat hemidiaphragms incubated anaerobically*

Bicarbonate buffer at 37° for 50 or 90 min. Glucose concentration 2 mg./ml. Insulin concentration 8 μg./ml. Results given as means ± S.E.

	No. of rats	$q^{N_2}_{CO_2}$ (ml./g. wet wt./hr.) 20–50 min. Control	20–50 min. Insulin	50–90 min. Control	50–90 min. Insulin	Rate of glucose uptake (mg./g. wet wt./hr.) 0–90 min. Control	0–90 min. Insulin
Series A	13	1·31 ± 0·43	1·49 ± 0·43	0·67 ± 0·27	0·77 ± 0·20	2·78 ± 1·00	2·78 ± 0·75
Series B	19	2·15 ± 0·40	2·26 ± 0·62	1·40 ± 0·29	1·43 ± 0·46	3·74 ± 0·32	4·02 ± 0·31
Series C	20	1·60 ± 0·37	1·55 ± 0·37	—	—	4·06 ± 0·37	4·01 ± 0·35

Table 2. *Balance of metabolites during incubation of rat diaphragm in bicarbonate medium*

The figures represent the mean changes, ± S.E., expressed in μmoles/g. of tissue, during the whole of the experimental period. Conditions as in Table 1. The number of experiments is given in parentheses. 'Theoretical lactic acid' is obtained from (glucose + glycogen disappearance) × 2.

Disappearance of glucose Control	Disappearance of glucose Insulin	Disappearance of glycogen Control	Disappearance of glycogen Insulin	Lactic acid (theoretical) Control	Lactic acid (theoretical) Insulin	Lactic acid (found) Control	Lactic acid (found) Insulin	Estimated output of CO_2 Control	Estimated output of CO_2 Insulin
				20 min. experiments (6)					
10·4 ± 2·8	—	9·5 ± 0·7	—	39·8	—	26·0 ± 3·1	—	39*	—
	9·2 ± 2·0	—	9·5 ± 0·7	—	37·4	—	24·0 ± 2·2	—	36*
				50 min. experiments (14)					
19·1 ± 2·1	—	15·3 ± 0·7	—	68·8	—	36·4 ± 1·8	—	78·6 ± 4·6	—
	19·3 ± 1·9	—	14·7 ± 0·9	—	68·0	—	36·9 ± 2·5	—	79·2 ± 4·7
				90 min. experiments (19)					
31·2 ± 2·6	—	14·4 ± 0·8	—	91·2	—	71·3 ± 6·3	—	138·0 ± 6·3	—
	33·5 ± 2·6	—	14·2 ± 0·8	—	95·4	—	77·3 ± 5·4	—	141·0 ± 7·5

* These two values are the mean estimates of CO_2 production during the first 20 min. incubation, obtained by extrapolation from the observations of the 50 and 90 min. experiments.

The figures in the 'control' and 'insulin' columns are paired results obtained from the same experiment.

Table 3. *Comparison of anaerobic CO_2 production by rat diaphragm with bicarbonate loss from the incubation medium*

Incubation for 90 min. Experimental conditions as in Table 2.

Expt. no.	CO_2 production estimated manometrically (μmoles/g.) Control	CO_2 production estimated manometrically (μmoles/g.) Insulin	HCO_3^- loss from medium (μmoles/g.) Control	HCO_3^- loss from medium (μmoles/g.) Insulin
1	153·5	146	—	123
2	122	100	114	108
3	144	147	83	89
4	103	129	115	168
5	160	163	173	—
6	132	154	156	155
Mean	136	140	128	129

suggestion of an insulin effect. The results shown in Table 1, Series A, had suggested the possibility that the increase in glycolysis rate apparently caused by insulin might be due to the formation, in the insulin-treated muscle, of another acid in addition to lactic acid, since there was no difference in the rates of utilization of the lactate precursors glucose and glycogen in the two sets of muscles. In the later experiments, therefore, the lactic acid of the medium was measured chemically at the end of the experiment. Insulin was found not to affect the rate of lactic acid production (Table 2), but it was observed that the amount of lactic acid produced was much less than the theoretical value calculated from the disappearance of glucose and glycogen. It was of interest to compare the lactic acid production with the liberation of CO_2 from the medium, and the CO_2 production was corrected by extrapolation to allow for the equilibrium period. It was in reasonable agreement with the directly estimated loss of bicarbonate from the medium (Table 3). The mean total CO_2 liberation so obtained is shown in the last column of Table 2; it was larger than the actual lactic acid found in the medium and than the theoretical maximum of lactic acid obtainable from the glucose and glycogen catabolized by the muscle. Some acid or acids other than lactic acid

must have diffused into the medium during the incubation; the discrepancy cannot otherwise be explained. Pyruvic acid was shown by qualitative paper chromatography to be present in the medium after the incubation (Altmann *et al.* 1951), but no complete identification of the acids present was made.

confirmed, and it is evident that, at least in anaerobic conditions, the medium of incubation may have a considerable effect on the efficiency of insulin action. The effect of the incubation medium on the metabolism of the muscle was further emphasized by measurements of the anaerobic lactate output in a phosphate buffer (Table 5).

Table 4. *Glucose uptake of rat diaphragm incubated anaerobically in a phosphate medium*

Buffer of Stadie & Zapp (1947), glucose 2 mg./ml. Insulin 8 μg./ml. Gassed with N_2, incubated at 37°. The figures are the mean rates of uptake ±S.E., in mg./g./hr.

Length of incubation	No. of observations	Glucose uptake: Control	Glucose uptake: Insulin	Significance
Hemidiaphragms				
30 min.	7	4·12±0·73	4·68±0·86	$P>0·10$
60 min.	8	3·40±0·55	3·72±0·54	
120 min.	4	1·70±0·18	1·77±0·10	
Quarter diaphragms				
20 min.	10	3·80±0·66	4·86±0·65	For paired differences $t=2·8$, $P=0·02$
40 min.	10	2·59±0·15	2·58±0·17	

Incubation in a phosphate buffer

The results shown in Tables 1 and 2 provide reasonable evidence that insulin is without effect on glucose utilization in some anaerobic conditions. Walaas & Walaas (1952), on the other hand, found that in a phosphate buffer insulin increased the uptake of glucose by diaphragm muscle. The increase was statistically significant only when the incubation was stopped after 15 min. This report was checked in this laboratory, using the conditions described in the preceding section, except that a phosphate buffer (that of Stadie & Zapp, 1947) was substituted for the bicarbonate Ringer. The rate of glucose uptake was found to be higher in the insulin-treated muscles after 30 and 60 min. incubation, but the increase was not statistically significant. After 120 min. incubation the glucose uptake was the same with and without insulin (Table 4). Experiments of 15 min. duration were unsuccessful because the very small amount of glucose which had disappeared from the 2 ml. of medium could not be estimated accurately. (The same difficulty was found in the 20 min. incubations in bicarbonate medium reported in Table 2, but in those experiments the lack of insulin effect was confirmed by the estimations of the rate of glycolysis.)

To meet this difficulty quarter-diaphragms, which can be incubated in as little as 0·4 ml. of medium in the side arm of a Warburg flask, were used instead of hemidiaphragms. The other conditions remained the same. Insulin was now found to increase significantly the glucose uptake in a 20 min. incubation, but was without effect at 40 min. (see Table 4). The work of Walaas & Walaas is thus

Table 5. *Lactate output by hemidiaphragms incubated anaerobically in a phosphate buffer, compared with disappearance of glucose and glycogen*

Incubation for 60 min., conditions as in Table 4. Values in μmoles/g. of tissue, ±S.E. Theoretical lactic acid = (glucose + glycogen disappearance) × 2.

No. of expts.	Lactate output	Lactic acid (theoretical)
6	84·1±6·5	83·9±6·4

Concentration of ATP in diaphragm muscle

Although exclusion of oxygen is probably the most easily controllable treatment by which the rate of ATP synthesis in the cell is likely to be reduced, other treatments have been used on the isolated diaphragm. Villee, Deane & Hastings (1949) added dinitrophenol (DNP) to the incubation medium, and reported that at a DNP concentration of 10^{-4}M insulin action was abolished, although glucose uptake continued, but at a lower rate than normal. Ottaway (1950) found that insulin action disappeared if the diaphragm was cut into thin strips, each weighing about 10 mg. Glucose utilization was not seriously diminished unless every fibre was damaged by the cutting.

It was of interest to determine whether there was any relationship between the concentration of ATP in the muscle after these treatments and the effectiveness of insulin, although it was realized that in a functioning cell the concentration of a metabolite is not necessarily related directly to the rate at which it is formed or removed. The results of the analyses are shown in Table 6, which also includes values of ATP concentration in diaphragm

Table 6. *Comparison of ATP concentration with effectiveness of insulin for rat-diaphragm muscle incubated in various conditions*

Concentrations are given in μmoles of ATP/g. wet wt. of tissue ±S.E. The effectiveness of insulin was estimated subjectively; see text.

Treatment	Length of incubation (min.)	No. of expts.	Effectiveness of insulin	ATP concn. (μmoles/g. wet wt.)
Hemidiaphragms, dissected only	0	6	.	3·89±0·13 (*a*)
Hemidiaphragms, phosphate, O_2	30	7	+ + +	3·65±0·35
Hemidiaphragms, phosphate, O_2	90	4	+ + +	2·96±0·15 (*b*)
Hemidiaphragms, bicarbonate, O_2+CO_2	30	8	+ + +	1·92±0·22 (*c*)
Hemidiaphragms, phosphate, O_2, DNP 10^{-5}M	30	5	+ +	1·79±0·18 (*c*)
Hemidiaphragms, phosphate, N_2	30	9	+	1·31±0·13 (*d*)
Hemidiaphragms, bicarbonate, N_2+CO_2	30	9	0	1·16±0·09 (*d*)
Quarter diaphragms, phosphate, N_2	30	6	+	0·97±0·05 (*e*)
Diaphragm strips, phosphate, O_2	30	5	0	0·96±0·10
Hemidiaphragms, phosphate, O_2, DNP 10^{-4}M	30	6	0	0·12±0·09 (*f*)

Tests of significance: the values marked by a letter in brackets are significantly different ($P<0·05$) from the values marked by the preceding and following letters.

immediately before incubation and after a normal aerobic incubation. The table shows that after 30 min. aerobic incubation in a phosphate buffer the concentration of ATP in the muscle had not decreased significantly, and even after 90 min. the concentration was only 25% below the initial level. After 30 min. aerobic incubation in a bicarbonate buffer, on the other hand, the concentration of muscle ATP had dropped to 50% of the initial concentration, but there has been no suggestion that insulin is less effective in bicarbonate-buffered media than in phosphate. Indeed, such media have been used (e.g. by Randle, 1954, and others) for the *in vitro* assay of insulin. The effectiveness of insulin in the other experimental conditions (estimated subjectively from the size and duration of the increase of glucose uptake which it caused) was roughly proportional to the concentration of ATP after 30 min. incubation, but the correlation was by no means good.

The minimum amount of hydrolysable P which could be detected was about 0·5 μg., equivalent to 0·2 μmole ATP/g. of tissue. In four of the six experiments with 10^{-4}M DNP, no hydrolysable P could be detected, and the concentration in the muscle was probably zero, although this cannot be stated with certainty.

DISCUSSION

Three conclusions may be drawn from these results. First, the insulin effect on diaphragm incubated anaerobically in a phosphate buffer lasts only for 15–30 min. This confirms the report of Walaas & Walaas (1952). Haft & Mirsky (1952) and Demis & Rothstein (1954) preincubated the diaphragms for 5–15 min. in nitrogen before adding insulin and glucose, and found no effect of insulin on glucose uptake. Demis & Rothstein concluded that this supported the findings of Walaas & Walaas. It may be of significance, in connexion with this fleeting insulin effect, that almost all the muscle glycogen has broken down after 30 min. anaerobic incubation (Ottaway, 1950). Thus after this interval the amount of substrate available for glycolysis must be diminished by almost 50%, since the rate at which glucose is taken up from the medium remains relatively constant.

Secondly, no trace of insulin effect on glucose uptake was found when the diaphragm was incubated anaerobically in a bicarbonate buffer. This difference may be associated with the relative efficiencies of glycolysis in the two media, as judged by the ratio of carbohydrate disappearance to lactate production (Tables 2 and 5). Thirdly, the ATP concentration in the muscle cells remained above 10^{-3}M for at least 30 min. in all the conditions examined, excepting only incubation with 10^{-4}M DNP.

The current hypotheses of the mechanism of insulin action outlined earlier in this paper may now be briefly discussed in the light of these findings. The possibility of a direct activation of hexokinase by insulin is not supported by the fact that the ATP concentration in the muscle remains relatively high after sensitivity to insulin has disappeared. Further, insulin has been shown to increase the rate of uptake by diaphragm of glucose, mannose (Liébecq, 1951), fructose (Haft, Mirsky & Perisutti, 1953; Mackler & Guest, 1953) and galactose (Demis & Rothstein, 1954). Wick & Drury (1953) have shown that insulin increases the rate of utilization of galactose by the eviscerate animal. Thus it appears unlikely that insulin activates a single enzyme, and indirect activation of a number of hexokinases becomes more probable.

Until a detailed mechanism to explain active transfer and the 'permeability barrier' in the

muscle cell has been put forward, it cannot be definitely stated that the results presented here are not in agreement with the second hypothesis of insulin action. There seems, however, no reason why a reduction of the 'permeability barrier' should not have taken place in the conditions of these experiments, since a supply of energy for transfer remained in the muscle in the form of ATP, and the rate of transfer of glucose could still be increased by increasing the glucose concentration. It must be borne in mind also that if the active transfer of glucose is enzymic, to postulate a reduction in the 'permeability barrier' of the cell may be only another way of stating that insulin activates an enzyme concerned with glucose transfer.

The insulin effect on the isolated diaphragm is abolished, while the normal glucose uptake remains unchanged, by four treatments of the muscle which have been investigated: anaerobiosis, severe damage, incubation with DNP or with iodoacetate (Haft & Mirsky, 1952). In three of these conditions it is well known that oxidative phosphorylation is impaired or abolished; after severe damage to the muscle it can be shown that the oxygen consumption rises and the ATP concentration falls (Ottaway, 1950). Demis & Rothstein (1954) make the point that the insulin effect on diaphragm persists for 15–30 min. after the muscle has been made anaerobic, and only reappears 15–30 min. after oxygen is readmitted, and they suggest that a reservoir of some material synthesized only in aerobic conditions must be present in the diaphragm for insulin to be effective. These facts support the hypothesis that insulin increases the rate of oxidative phosphorylation, or of some closely allied process.

If, however, the hypothesis is stated in the more usual form that insulin increases the rate or efficiency of ATP resynthesis, serious objections can be made to it. The poor correlation between ATP concentration and effectiveness of insulin has been touched on earlier in this paper. It is true that concentration of a substrate is often not a good guide to the rate at which it is produced, but in these experiments it is reasonable to expect some connexion between concentration of ATP and the rate at which it is produced. Then, it has never been clear why an insulin effect on the efficiency of oxidative phosphorylation should alter the activity of hexokinase *in vivo*, because the ATP concentration is maintained well above the saturation level of the enzyme (see Table 6; also Mommaerts, 1951). A differentiation is also implied by this hypothesis between ATP synthesized in oxidative phosphorylation and that synthesized in glycolysis. There is little clear-cut evidence for this. Finally, the 'reservoir of material synthesized only in aerobic conditions', postulated by Demis & Rothstein, is unlikely to be ATP, because any adenylic acid formed during an anaerobic period will almost certainly be deaminated to inosinic acid and will not be available as a phosphate acceptor when oxygen is readmitted (Parnas, 1935). Of the phosphate esters other than ATP which have been studied in isolated diaphragm, phosphocreatine has been shown to disappear very quickly in anaerobic conditions (Walaas & Walaas, 1952) or on treatment with DNP (Sacks & Sinex, 1952). This compound has not been considered to be directly implicated in the utilization of glucose (see, however, a discussion by Sacks, 1952, and by Handler, 1952; Ord & Stocken, 1955). Further examination of the relation between sensitivity to insulin and the phosphate esters of muscle would probably throw more light on the mechanism of insulin action.

SUMMARY

1. The effect of insulin on glucose utilization by the isolated diaphragm of the rat, incubated in anaerobic conditions, has been studied.

2. Insulin was found to increase the rate of uptake of glucose by diaphragm incubated for 20 min. in a phosphate buffer. On longer incubation the effect of insulin ceased to be significant. No effect of insulin was found, either on glucose uptake or on glycolysis, when the diaphragm was incubated in a bicarbonate buffer.

3. The rates of disappearance of glucose and glycogen, and of formation of lactic acid and carbon dioxide, were studied in diaphragm incubated in bicarbonate. Lactate production was found to be substantially less than carbohydrate utilization and total acid production. Pyruvic acid was identified in the medium after incubation.

4. The concentration of adenosine triphosphate in diaphragm muscle was estimated after incubation under various conditions. The concentration was found to remain above saturation level for hexokinase for at least 30 min., in all conditions except incubation with 10^{-4} M dinitrophenol.

5. The relationship between these findings and possible mechanisms of insulin action is discussed.

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The Effect of Vitamin A Deficiency on the Cholesterol Levels of the Plasma and Liver of the Rat

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Although much research has been devoted to various aspects of the chemistry and biochemistry of vitamin A, the systemic mode of action remains an unsolved problem.

Lowe, Morton & Harrison (1953) showed that an unidentified and perhaps hitherto unknown substance accumulates in the livers of vitamin A-deficient rats. A working hypothesis was put forward to account for this, namely, that vitamin A exerts a restraining influence on the dehydrogenation of cholesterol or steroid hormones, such restraint being lifted in vitamin A deficiency, with consequent appearance of an abnormal product or products. The new substance is, however, quantitatively only a minor constituent, and it becomes important to know whether the aberrant metabolism to which it provides a clue can result in large changes in major constituents.

Cholesterol is itself probably a precursor of most steroid hormones, and fresh significance may attach to the question as to whether or not the more active sites of cholesterol metabolism suffer marked changes in vitamin A deficiency. Several groups of workers have tried to follow plasma cholesterol levels during the development of vitamin A deficiency. Ralli & Waterhouse (1933) reported a rise in the blood-cholesterol level of four dogs maintained on a vitamin A-free diet, but the level fell somewhat in two of the dogs as the deficiency symptoms became severe. Administration of carotene or cod-liver oil to two of the vitamin A-deficient dogs produced a marked fall in the blood cholesterol to normal levels. Smith (1934) claimed that the blood cholesterol level of rats maintained on a vitamin A-free diet was higher than that of control animals, but that in the final stages of deficiency the level became lower than that of the controls. This observation was supported by the work of Collazo, Torres & Sanchez-Rodriguez (1934), who observed that blood from rats exhibiting the full picture of vitamin A deficiency had a much lower cholesterol content than blood from control animals. More recently, Mayer & Truant (1949) referred to unpublished work by Mayer indicating a 'piling-up' of cholesterol (presumably in the plasma) in vitamin A deficiency. On the other hand, Sure, Kik & Church (1933) were unable to find any changes in blood cholesterol of rats during the development of vitamin A deficiency even to the stage of severe xerophthalmia and marked loss of weight. The evidence is conflicting and there is clearly need to reinvestigate the matter.

In the present work, particular attention has been given to the experimental design so that the results could be subjected to a satisfactory statistical analysis. Concurrently, a study of the liver cholesterol content of vitamin A-deficient and control rats was undertaken.

EXPERIMENTAL

Materials

Vitamin A solution. The vitamin A administered to the control rats was in the form of a tested cod-liver oil diluted with arachis oil, so that the daily dose per rat (12 or 20 i.u.) was contained in 0·1 ml. of the solution.

Animals and diets. Newly weaned hooded rats were placed on animal diet no. 4 (British Extracting Co. Ltd., Bromborough Port, Cheshire) until about 40 days old (wt. 60–100 g.); this allowed them to store moderate amounts of vitamin A. The animals were then transferred to a vitamin A-free diet made up of casein (Genatosan low vitamin content) 18%; maize starch, 65%; arachis oil, 5%; Marmite, 8%; salt mixture (Osborne & Mendel, 1913), 4%. Vitamin E was not added (see p. 452).

Procedure. Male and female rats from six litters were divided by card selection into two groups (I and II); each group contained twenty rats, and was arranged so that for each rat in group I there was a corresponding litter mate of the same sex in group II, there being equal numbers of males and females in each group. The animals were housed separately throughout the experiment, the cages being arranged so that each subgroup of four rats in either group consisted of two males and two females. Each animal received 10 g. of diet/day for the first week and 12 g./day thereafter. Water was freely provided. The rats in group I received, in addition, 12 i.u. vitamin A/day until day 113, when the daily dose was increased to 20 i.u. until the end of the experiment. The animals in group II received 0·1 ml. of arachis oil to balance the amount given to those in group I. Both vitaminized arachis oil and arachis oil were administered orally from a pipette. The animals grew well, and on the 58th day of the experiment fourteen rats receiving 12 i.u./day of vitamin A had increased in weight by 1448 g. (average 103·4), whereas sixteen rats not receiving vitamin A had increased in weight by 1595 g. (average 99·7 g.).

Analyses of blood and tissues

Frequency of plasma-cholesterol determinations. Determinations were performed on eight rats each day (one set of four from each group) so that the forty animals were examined in 5 consecutive days. Analyses were carried out on the same eight rats at intervals of 14 or 21 days throughout the experiment. When a rat in group II was xerophthalmic and losing weight it was killed, together with the corresponding rat from group I. The rats were anaesthetized with $CHCl_3$ and as much blood as possible was withdrawn by cardiac puncture (Glover, Goodwin & Morton, 1947). Final duplicate cholesterol determinations were made on each plasma.

Routine collection of blood samples. Blood (0·5 ml.) was withdrawn by cardiac puncture from each rat. With this technique there is always the risk that the animal may die from shock or haemorrhage. In our experiments 212 punctures were performed and there were five deaths from haemorrhage and six from shock. Nevertheless, this procedure for obtaining samples of blood at intervals from the same rat was found to be preferable on all counts to other methods such as severing the tip of the tail. The heparinized blood was transferred to an ignition tube fitted with a rubber collar. This was then placed inside a 15 ml. centrifuge tube and the assembly centrifuged. The plasma was carefully removed by means of a capillary pipette and transferred to a watch-glass.

Estimation of cholesterol. The method employed was a modification of that described by Kenny (1952). Plasma (0·2 ml.) was pipetted into 5 ml. of ethanol–ether mixture (3 vol. of 95% ethanol: 1 vol. of ether) contained in a 10 ml. glass-stoppered test tube; the tube was then stoppered and shaken to mix the sample thoroughly. After loosening the stopper, the tube was heated in a boiling-water bath until the mixture just boiled. The stopper was replaced firmly and the tube and contents were shaken vigorously for about 4 min. The mixture was centrifuged (1800 *g* for 10 min.), after which the supernatant ethanol–ether mixture was decanted into a boiling tube. The precipitate was resuspended in 5 ml. of ethanol–ether mixture, heated just to boiling, shaken for 4 min. and recentrifuged. The supernatant was again decanted off completely and added to the first 5 ml. of extract. A portion (8 ml.) of this ethanol–ether extract was transferred to a boiling tube, a piece of capillary tubing sealed at the upper end was added to prevent bumping and the contents were evaporated to dryness in a boiling-water bath. The tube was then placed in an electric oven at 100° for 10 min. to remove the last traces of ethanol and water; after cooling, the residue was extracted with 7 ml. of $CHCl_3$, the walls of the tube being scraped with a glass rod to remove any adhering particles. The solution was warmed on a water bath and quickly filtered through a Whatman no. 1 filter paper, the top of the funnel being covered with a watch glass to minimize evaporation. A portion (5 ml.) of the filtrate was transferred to a clean, dry glass-stoppered tube and 1 ml. of acetic anhydride was added. The tube was then placed in a water bath at 37° for 3 min. to allow temperature equilibration to occur, after which 0·1 ml. of H_2SO_4 (A.R.) was added from a micro-burette, the stopper was replaced and the contents of the tube were mixed by shaking. The tube was then incubated in the water bath at 37° for 30 min., light being rigorously excluded during this period. The extinction of the solution at 410 mμ. was then measured in a Unicam S.P. 600 spectrophotometer, using a blank prepared as described below. The amount of cholesterol present in the solution was calculated from a standard curve prepared from solutions containing known amounts of cholesterol.

Blank and standard. Cholesterol solution (5 ml.) [0·005% (w/v) in $CHCl_3$ freshly prepared from a stock solution of 0·02% (w/v) cholesterol (in $CHCl_3$)] was evaporated to dryness in a glass-stoppered tube; 10 ml. of ethanol–ether mixture were added, heated just to boiling, shaken and allowed to cool. Part of this solution (8 ml.) was transferred to a boiling tube and evaporated to dryness as for the test. Both were then treated exactly as described for the plasma extract, except that with the blank the H_2SO_4 was omitted. As volumes of plasma in excess of 0·2 ml. could not be obtained with safety, it was necessary to use 'reagent blanks' in place of true 'plasma blanks'. Comparison of these two types of blank revealed that the extinction values were almost identical. No allowance has been made for the higher extinction given by cholesterol esters in this method (see Kenny, 1952).

In our experience, when estimating small amounts of cholesterol (of the order of 0·15–0·2 mg.), it is essential to adhere strictly to this experimental procedure. This method gives results reproducible to within 5%.

Livers and kidneys

Extraction of lipids. At the end of the experiment the livers and kidneys were removed, washed, dried on filter paper and weighed. The kidney lipid was extracted and the vitamin A content determined by the method of Glover *et al.* (1947).

For two rats (I_1 and II_1) the liver lipid was extracted by the method of Glover *et al.* (1947). Parts of the livers from rats (I_3 and II_3) were examined in the Pathology Department; the lipid was extracted from the remainder by the method of Glover *et al.* (1947), except that 95% ethanol was used as the solvent instead of ether.

Saponification of livers. Most of the livers were directly saponified by the following method: to the moist tissue 60% (w/v) KOH (0·5 ml./g.) was added and digestion continued on a water bath until the mixture was homogeneous. Ethanol (2 vol.) was then added and the mixture boiled for a further 30–40 min. under reflux. The mixture was extracted six times with ether (freshly distilled over reduced iron); the extracts were bulked and washed with water until free of alkali, dried over anhydrous Na_2SO_4, filtered through a sintered-glass funnel (G3), evaporated to dryness under N_2 and weighed. The lipids from the livers of rats I_1, II_1, I_3 and II_3 were saponified by this method before cholesterol determinations.

Determination of liver cholesterol. The unsaponifiable matter was dissolved in a suitable volume of $CHCl_3$ and 5 ml. portions were transferred to each of three glass-stoppered tubes (one blank, two tests). The cholesterol content was then determined by adding 1 ml. of acetic anhydride and proceeding as already described under 'Estimation of cholesterol'.

RESULTS

When the rats were killed at the close of the experiment, gross abnormalities of the liver, urinary bladder and testes were seen in several vitamin A-deficient animals. The livers of II_1, II_3 and II_5 presented signs of fatty infiltration, since they were distinctly yellow and friable. Portions of livers I_3 and II_3 were examined histologically, and it was found that the liver of rat II_3 was indeed infiltrated with fat, whereas that of I_3 (control) was normal. The remaining portions of these livers, and the other fatty livers, were examined as usual and the results are included in the analyses. Grossly distended urinary bladders were observed in rats II_6, II_8 and II_{15}, and the testes of rats II_{16} and II_{18} were abnormally small and discoloured. All the organs of the control rats appeared normal.

The complete series of plasma cholesterol values as determined at intervals throughout the experiment, together with the terminal plasma vitamin A levels, are presented in Table 1. The mean and standard deviation for each set of values are recorded at the foot of the respective columns.

The liver cholesterol contents for both groups of rats, together with the kidney vitamin A levels, are recorded in Table 2. Results of statistical analyses of sex differences in the liver cholesterol contents of the two groups are given in Table 3.

DISCUSSION

In order to ensure a long depletion period, the rats were maintained on the stock colony diet until they were about 40 days old; they were then placed on the vitamin A-free diet. For some rats the depletion lasted for 19 weeks, and in all animals it was long enough to allow six determinations of plasma cholesterol to be made in each survivor, together with a final duplicate determination when signs of vitamin A deficiency (severe loss of weight and xerophthalmia) were exhibited.

The kidneys from each rat were examined for vitamin A (Table 2) because at low levels of vitamin A intake the kidney exceeds the liver in its capacity to store the vitamin (Johnson & Baumann, 1947).

From Table 1 it can be seen that there was no significant difference (1% level) between the mean plasma cholesterol levels of the deficient and control animals at any time during the experiment. Moreover, statistical analysis (Table 2) of the liver cholesterol (whether expressed as total cholesterol/liver or as mg. cholesterol/g. moist liver) reveals that there was again no significant difference between the two groups ($P = 0{\cdot}05$).

The results of Lowe *et al.* (1953) suggested the possibility that cholesterol metabolism was drastically deranged in vitamin A deficiency. Such a derangement could be pictured as a sequence of abnormal metabolites, only one of which could be detected by ultraviolet spectrophotometry; in the aggregate a substantial proportion of the steroid content might be affected. Alternatively the deranged metabolism might be quantitatively of little significance in the sense that the proportion of steroid molecules affected would be quite small, although the new materials might be qualitatively sharply different, e.g. in their physiological effects. The conflicting reports on the behaviour of plasma cholesterol levels in vitamin A deficiency do not permit a decision to be made between these alternatives.

Plasma cholesterol levels are profoundly affected by alterations in the rate of conversion and destruction of cholesterol (Byers, Friedman & Rosenman, 1952). In our experiments the synthetic diet was virtually sterol-free. Hence, the fact that there is no significant difference in the plasma and liver cholesterol levels between the two groups indicates that no quantitatively major alteration in endogenous cholesterol metabolism occurs during vitamin A depletion. If therefore the abnormal metabolite which appears in the livers of vitamin A-deficient rats is indeed a dehydrogenation product of cholesterol or of steroid hormones, the amounts of this compound are seen to be too small to make a demonstrable contribution to the

Table 1. *Serial plasma cholesterol and terminal plasma vitamin A levels in vitamin A-deprived (G II) and control (G I) rats*

		Plasma cholesterol (mg./100 ml.)														Terminal vitamin A (i.u./100 ml.)	
		Week 1		Week 3		Week 5		Week 7		Week 10		Week 13		Terminal			
Rat	Sex	G I	G II	G I	G II	G I	G II	G I	G II	G I	G II	G I	G II	G I	G II	G I	G II
1	M.	106	103	103	116	102	103	103	103	89	111	98	112	80	116	73·4	Nil
2	M.	90	107	103	102	90	—	102	—	102	—	97	—	88	—	53·7	—
3	F.	—	128	114	113	120	109	120	102	114	108	103	74	125	157	109	Nil
4	F.	146	92	137	97	119	120	138	112	—	—	—	—	—	—	—	—
5	M.	107	136	107	125	114	115	100	118	109	102	104	114	89	137	—	Nil
6	F.	141	113	136	127	121	130	131	108	—	112	—	103	—	77	—	Nil
7	M.	103	131	107	133	102	113	97	114	86	114	103	124	101	112	—	Nil
8	F.	129	149	114	130	103	141	119	138	137	144	98	126	100	132	45·8	Nil
9	M.	131	125	114	114	123	103	108	113	—	84	—	90	—	—	—	—
10	F.	115	121	129	125	123	102	129	83	109	—	111	—	103	—	59·8	—
11	M.	143	120	155	102	—	102	—	102	—	87	—	121	—	—	—	—
12	F.	143	127	154	—	132	—	141	—	107	—	115	—	111	—	85	—
13	F.	111	121	114	102	107	114	129	103	113	141	125	95	96	58	48·5	Nil
14	M.	119	114	119	114	121	120	118	108	114	131	—	115	—	92	—	Nil
15	F.	—	108	109	125	117	103	114	117	107	90	95	82	94	37	120	Nil
16	M.	88	107	—	131	109	100	88	98	91	97	81	71	76	69	52·7	Nil
17	F.	109	100	111	131	102	147	120	131	129	124	121	80	115	52	80	Nil
18	M.	105	114	98	143	127	129	103	125	131	137	102	89	114	85	101	Nil
19·	M.	124	114	109	108	119	113	—	109	—	103	—	126	—	—	—	—
20	F.	—	113	—	132	—	124	—	120	—	121	—	82	—	103	—	Nil
Mean		118·2	117·2	118·5	119·5	113·9	116·0	115·3	111·3	109·8	112·9	104·1	100·3	99·4	94·4	—	—
S.D. ±		18·32	13·29	16·9	13·1	11·0	13·9	15·2	12·7	15·28	18·94	11·68	19·58	14·33	35·88	—	—
F				0·039		0·243		0·698		0·223		0·384					
Required for significance (1%)				7·42		7·44		7·47		7·64		7·68					

Table 2. *Liver cholesterol and kidney vitamin A levels in vitamin A-deficient (group II) and control (group I) rats*

Rat	Sex	Total liver cholesterol (mg.)		Liver cholesterol (mg./g. tissue)		Kidney vitamin A (i.u./g.)	
		Group I	Group II	Group I	Group II	Group I	Group II
1	M.	32·72	32·08	3·976	3·565	9·8	Nil
2	M.	18·88	—	2·289	—	7·0	—
3	F.	10·08	35·33	2·045	4·087	4·9	Nil
5	M.	16·35	33·36	2·296	3·848	—	—
6	F.	—	20·00	—	3·378	—	—
7	M.	29·10	13·92	4·163	2·611	12·6	Nil
8	F.	16·16	17·20	2·887	3·532	3·5	—
10	F.	13·84	—	2·057	—	5·0	—
12	F.	13·04	—	1·836	—	7·8	—
13	F.	16·64	11·40	2·586	1·786	9·5	Nil
14	M.	—	24·40	—	4·014	—	Nil
15	F.	16·30	14·64	2·358	1·920	8·8	Nil
16	M.	22·96	11·80	2·375	1·825	8·2	Nil
17	F.	16·01	7·79	2·185	1·306	4·5	Nil
18	M.	19·28	14·80	2·180	2·210	8·5	Nil
20	F.	—	17·50	—	3·301	—	Nil
Mean		18·57	19·56	2·556	2·876	—	—
S.D.		±6·34	±9·01	±0·660	±0·967	—	—
	t (Found) =	0·3251		0·9552			
	t (Tables) =	2·064		2·064			

Level of probability = 0·05.
For significance t (found) must exceed t (tables) at the desired level of probability (Snedecor, 1946).

Table 3. *Comparison of liver cholesterol in vitamin A-deficient (group II) and control (group I) rats*

		Total cholesterol (mg.)	t (found)	t (tables)	Concentration (mg./g. tissue)	t (found)	t (tables)
Males	Group I	23·215±6·426*	0·317	2·228	2·880±0·926*	0·494	2·228
	Group II	21·727±9·562			3·012±0·919		
Females	Group I	14·58 ±2·412	0·9025	2·179	2·279±0·360	1·129	2·179
	Group II	17·69 ±8·791			2·759±1·064		
Group I	Males	23·215±6·426	3·534	2·201	2·880±0·926	1·675	2·201
	Females	14·58 ±2·412			2·279±0·361		
Group II	Males	21·727±9·562	0·794	2·201	3·012±0·919	0·4542	2·201
	Females	17·69 ±8·791			2·759±1·064		

* S.E.M.

For significance t (found) must exceed t (tables) at the desired level of probability (taken as 0·05) (Snedecor, 1946).

total liver and plasma cholesterol levels. If this compound is itself toxic or is the precursor of other toxic substances which, carried to other parts of the body, induce the derangement of normal epithelial metabolism so characteristic of the vitamin A-deficiency state, then the amount of this compound formed need only be relatively small. For these reasons, the results reported here are not necessarily incompatible with the working hypothesis, previously advanced, about the systemic mode of action of vitamin A.

Closer study of Table 1 reveals that the mean value for each set of plasma cholesterol determinations in both groups I and II undergoes a gradual decline during the experimental period. This observation is not unexpected, since the animals were on a diet virtually free of sterols. In general, the plasma cholesterol levels of the vitamin A-deprived rats showed only minor fluctuations before the very late stages of the deficiency. When the rats in group II were avitaminotic they could be subdivided into three groups according as the plasma cholesterol tended to rise, fall or remain constant. In rats 3 and 5 there was a sharp rise, in 13, 14, 15, 17 and 18 a severe fall (in 6, 9 and 16 the decline was more gradual), and in 1, 7, 8 and 20 there was little change in plasma cholesterol values.

In view of these variations, it might be possible to reach erroneous conclusions if a small number of rats were used in the experimental design. It is therefore interesting to note that our findings on

plasma cholesterol levels confirm those of Sure *et al.* (1933), who used thirty-four test and twenty-seven control rats, but they are not in agreement with those of Collazo *et al.* (1934), Ralli & Waterhouse (1933) and Smith (1934), none of whom used more than six test animals.

Our results on liver cholesterol content are in full agreement with those of Gillum & Okey (1936*a*) but not with those of Javillier, Rousseau & Emerique (1929) and Collazo *et al.* (1934). These last two groups of workers claimed that the livers of vitamin A-deficient animals had a lower cholesterol content than those of controls. However, since liver cholesterol levels are affected by dietary sterols, it should be noted that although Gillum & Okey (1936*a*) employed a sterol-free diet, Collazo *et al.* (1934) used such a diet for the deficient animals but not for the controls, whereas the diet employed by Javillier *et al.* (1929) was not fully reported.

Rats II_1, II_3 and II_5, which all had fatty livers, merit special mention. In II_3 and II_5, the plasma cholesterol levels rose sharply, but in II_1 no marked change was observed, although all three livers had a high cholesterol content (Table 2). In this Laboratory, fatty livers have been observed previously in several of our vitamin A-deficient rats, and it would appear that in the vitamin A-deficient state the liver is rendered more susceptible to fatty infiltration. When tocopherol was added to the vitamin A-deficient diet the abnormalities recorded in the present paper were observed (unpublished work). It is interesting to note that the plasma cholesterol levels of the control rats lie within a fairly restricted range, whereas the terminal liver levels exhibit marked variation. The deficient rat plasma cholesterol levels remain fairly constant until the terminal stages are reached, when they become very unstable. The terminal liver levels of these rats, however, vary even more widely than the terminal plasma levels (see S.D. values, Tables 1 and 2).

Since sex hormones are known to influence cholesterol metabolism (see below) and distribution of vitamin A (Moore, Sharman & Ward, 1951), it seemed of interest to compare the liver cholesterol contents of group I males with those of the males in group II, a similar comparison being made of the females of the two groups. However, in neither case did statistical analysis reveal any significant difference, whether the cholesterol was expressed as mg./g. of moist liver or as total/liver (Table 3).

Our results show that the livers of the control male rats contained a higher absolute amount of cholesterol than the livers of the control females, this difference being statistically significant (Table 3) ($P = 0{\cdot}05$). However, there was no significant difference when a similar comparison was made on the vitamin A-deficient animals. These findings confirm those of Okey, Gillum & Yokela (1934) and Gillum & Okey (1936*b*); moreover, like these two groups of workers and like Chamberlain (1929), we found that when the liver cholesterol was expressed as mg./g. moist liver the mean figure for the control male rats was higher than the mean for the control females, but this difference was not statistically significant (Table 3).

Although the livers of the control females were on average lighter than those of the males (6·435 g. female and 8·18 g. male) and the male livers tended to decline in weight during vitamin A deficiency, whereas those of the females did not (6·387 g. female, 7·041 g. male), it is unlikely that the differences in liver cholesterol can be due entirely to the weight factor. It seems probable that a full explanation must include an influence exerted by sex hormones. Since androgens have little effect on plasma cholesterol levels (Entenman, Lorenz & Chaikoff, 1940; Rosenman, Friedman & Byers, 1952) or on the rate of hepatic cholesterol synthesis (Rosenman *et al.* 1952), the sex differences found in the liver cholesterol content of the normal rats cannot be explained as an effect of the male sex hormones. On the other hand, although oestrogens do not readily affect plasma cholesterol levels in the rat (Horlick & Havel, 1948; Rosenman *et al.* 1952), they do decrease the hepatic synthesis of cholesterol (Rosenman *et al.* 1952). Since our diet had a negligible sterol content (a factor favouring endogenous sterol production), the effect of oestrogens on hepatic cholesterol synthesis might be exaggerated, and in consequence the difference in the liver cholesterol content of male and female control animals would be at least partially accounted for.

SUMMARY

1. A modified method for the estimation of cholesterol in small volumes of plasma is described.

2. By the use of this method, plasma cholesterol levels were determined at intervals on rats undergoing depletion of vitamin A and on their littermate controls.

3. At the end of the depletion period, when the deficient animals exhibited severe xerophthalmia and loss of weight, total liver cholesterol contents were determined on both groups of rats. There was no significant difference between the plasma cholesterol levels of the deficient and control rats at any stage during the depletion, neither was there any difference between the terminal liver cholesterol contents of the two groups.

4. The results are discussed in relation to earlier work and it is concluded that in a major and quantitative sense cholesterol metabolism is not responsive to vitamin A depletion.

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The Isolation of *n*-Pentadecanoic and *n*-Heptadecanoic Acids from Shark (*Galeorhinus australis* Macleay) Liver Oil

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Although the occurrence in natural fats of normal odd-numbered fatty acids, including *n*-pentadecanoic and *n*-heptadecanoic acids, has been reported by various investigators, subsequent work has invariably shown that the acids described were in fact mixtures of adjacent members of even-numbered fatty acids. The generally accepted view until recently is indicated by Hilditch (1947) as follows: 'with the solitary exception of *iso*-valeric acid (found only in the depot fats of the dolphin and porpoise) the molecules of all natural straight-chain fatty acids, saturated or unsaturated, contain an even number of carbon atoms.' This view, however, now requires modification as, after the isolation of pure *n*-heptadecanoic acid from hydrogenated mutton fat (Hansen, Shorland & Cooke, 1954*a*, *b*), *n*-pentadecanoic acid has been found in hydrogenated mutton fat (Hansen, Shorland & Cooke, 1954*c*), in hydrogenated shark-liver oil (Morice & Shorland, 1954) and in butterfat (Shorland, Gerson & Hansen, 1955). In addition, *n*-undecanoic acid was shown to be present in hydrogenated butterfat (Hansen, Shorland & Cooke, 1955); also, by means of liquid–gas partition chromatography (James & Martin, 1952), Hansen & McInnes (1954) demonstrated the occurrence in hydrogenated ox tallow of all the odd-numbered carbon acids from C_3 to C_9 inclusive.

In this paper is described the isolation of *n*-pentadecanoic and *n*-heptadecanoic acids from shark-liver oil by the combined processes of fractional distillation and crystallization without recourse to hydrogenation.

EXPERIMENTAL

The X-ray spacings were determined with a Philips Geiger X-ray spectrometer, Fe-filtered Kα radiation being used. The samples were melted on a glass slide and quickly cooled. Melting points are uncorrected. The specific rotations were determined on pure fractions, except where these melted above 24°. Such fractions were diluted with $CHCl_3$. C and H analyses were by Drs G. Weiler and F. B. Strauss, Oxford.

The oil (saponification equiv. 320·0, iodine value 161·9, unsaponifiable matter 8·4%, acid value 5·2) was prepared by solvent extraction (cf. Shorland, Bruce & Jessop, 1952) of the fresh livers of thirty-one male sharks caught in Cook Strait on 21 June 1944. The crude methyl esters prepared from the oil were distilled under high vacuum (at approx. 0·001 mm.) in a falling-film still at 140–160°, the residue being recycled twice to separate the volatile methyl esters

(6300 g.) from the non-volatile unsaponifiable matter. The final residue thus obtained weighed 800 g. and contained 45·5% unsaponifiable matter.

The methyl esters (6290 g.) were fractionated at 0·1 mm. in a Vigreux column (208 cm. × 6·5 cm.), giving ten fractions denoted A 1–A 10.

n-*Pentadecanoic acid*

Fractional distillation at approx. 0·1 mm. of A 2 (680 g., saponification equiv. 265·0, iodine value 31·2) in a column (150 cm. × 2·4 cm.) packed with stainless-steel gauze rings $\frac{1}{8}$ in. in diameter (Dixon, 1949) resulted in a series of 16 fractions (A 2, 1–16) of which those shown in Table 1 were used for the isolation of *n*-pentadecanoic acid, being subjected to systematic fractionation in column *E* (Shorland, 1952) whereby methyl esters of C_{14}, C_{15} and C_{16} acids were separated. In addition, as in the butterfat sample earlier examined by Shorland *et al.* (1955), the methyl esters of the C_{15} acids were resolved into low melting-point fractions possessing optical activity and high melting-point fractions from which optical activity was absent. These higher melting-point fractions were combined giving 21·5 g. of esters. The corresponding acids (14·0 g.) were crystallized at −40° three times from 40 vol. and once from 100 vol. light petroleum (b.p. 40–60°). The insoluble acids were again crystallized twice from 100 vol. acetone giving 10·2 g. precipitate denoted C, m.p. 52·5–53·0°. For further purification the acids were converted into methyl esters (10·7 g.) and fractionated in column *E* (Shorland, 1952), as shown in Table 2. The acids (1·41 g.) from C2 (Table 2) were crystallized from 20 vol. of acetone at −5°, giving 0·90 g. precipitate (C2I), m.p. 52·5–53·0° and a soluble fraction (C2S), 0·50 g., m.p. 50·5–51·0°. Of the acids from C3, 1·25 g. were similarly crystallized, but at −18°, resulting in 1·04 g. precipitate (C3I), m.p. 52·2–52·7°, and a soluble fraction (C3S), 0·21 g., m.p. 50·0–51·5°. C3I on admixture with authentic pentadecanoic acid, fraction O16S7S (cf. Hansen *et al.* 1954*c*), showed no depression in melting point.

Table 1. *Characteristics of fractions used for isolation of* n-*pentadecanoic acid*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value (Wijs)	M.p.
A 2, 4	27·2	243·6	6·4	15–16°
5	23·6	255·5	14·9	0–4
6	18·1	261·3	19·0	14–15
7	24·7	265·3	65·9	−10–3

Table 2. *Fractionation of methyl esters* (C) *at* 0·1 *mm.*

Fraction	Wt. (g.)	Saponification equiv.	M.p. Esters	M.p. Acids
C1	2·0	255·2	17·9–18·2°	51·0–51·5°
C2	2·4	256·4	18·5–18·7	51·5–52·5
C3	2·3	256·4	18·6–18·8	51·5–52·0
C4	4·0	257·7	18·0–18·2	51·5–52·5

The chemical and physical properties of fraction C3I were as follows: saponification equiv., 243·0; iodine value, 0·13; C, 74·1, H, 12·4% (calc. for $C_{15}H_{30}O_2$: saponification equiv., 242·4; iodine value, 0·0; C, 74·3; H, 12·5%), X-ray long spacing 35·55 Å, n_D^{60} 1·4328.

n-*Heptadecanoic acid*

To obtain a concentrate of methyl esters of C_{17} acids fractions A 3 (667 g., saponification equiv. 277·2, iodine value 36·9) and A 4 (637 g., saponification equiv. 286·9, iodine value 52·4) were refractionated. Fractions A 3, 10 and 11, and A 4, 13–16, whose equivalents approximated to 284, were combined with fractions 1 and 2 obtained from refractionation of A 3, 12, and A 4, 17 (108·8 g.) and denoted D. Crystallization of the acids (99·0 g.) from acetone, using 10 vol. four times at −78° and 15 vol. three times at −40°, gave 30·9 g. insoluble acids (DI). DI was converted into methyl esters (31·8 g.) and fractionated in column *E* (Shorland, 1952) at 0·1 mm. as shown in Table 3.

From previous experience with the isomers of pentadecanoic acid (cf. Shorland *et al.* 1955) it was assumed that methyl *n*-heptadecanoate would follow the methyl esters of the branched-chain fatty acid isomers during fractional distillation. This tendency is indicated by the sequence of the melting points (see Table 3), though the relative constancy of the optical rotation in the various methyl heptadecanoate fractions shows that the resolution of the isomers is incomplete. To isolate methyl *n*-heptadecanoate, therefore, fraction DI, 8 was converted into acids (4·5 g.) and crystallized at −40°, using successively 40 vol. acetone (twice), 100 vol. acetone and finally 40 vol. (twice) of ether. The final precipitate, denoted E (3·3 g.), melted at 60·5–61·0° (undepressed on admixture with authentic heptadecanoic acid), while the soluble portion (0·2 g.) melted at 53–55°. Further purification was effected as shown in Table 4 by fractional distillation at 0·1 mm. of the methyl esters.

Table 3. *Fractional distillation of methyl esters containing mainly* C_{17} *acids*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value (Wijs)	M.p.	$[\alpha]_D^{24}$
DI, 1	2·1	265·6	0·5	24–26°	0·08
DI, 2	3·4	268·7	0·2	29–30	0·12
DI, 3	5·0	273·5	0·9	24–25	0·12
DI, 4	5·3	284·6	1·5	10–11	0·38
DI, 5	1·9	283·3	1·6	9–10	0·44
DI, 6	5·0	284·4	0·9	10–12	0·48
DI, 7	3·4	283·1	1·9	15–19	0·40
DI, 8	5·4	293·9*	6·8	24–25	0·44

* Saponification equiv. of methyl esters freed from unsaponifiable matter, 285·3.

The acids from E2 (0·85 g.) and E3 (0·84 g.) were crystallized from 20 vol. of acetone at −5°, giving insoluble fractions E2I, 0·73 g., m.p. 60·5–61·5°, and E3I, 0·71 g., m.p. 61·0–61·3°. The soluble portions E2S, 0·12 g., and E3S, 0·13 g., melted respectively at 54–58° and 59·0–59·5°.

Table 4. *Purification of methyl* n*-heptadecanoate by fractional distillation*

Fraction	Wt. (g.)	Saponification equiv.	M.p.
E1	1·22	284·2	28·4–29·3°
E2	0·89	284·5	29·7–30·2
E3	0·90	286·7	29·2–30·2
E4	0·44	303·2*	29·7–30·1

* Saponification equiv. of methyl esters freed from unsaponifiable matter, 293·2.

The chemical and physical properties of fraction E3I were as follows: saponification equiv., 270·7; iodine value, 0·92; C, 75·4; H, 12·5% (calc. for $C_{17}H_{34}O_2$: saponification equiv., 270·4; iodine value, 0·0; C, 75·5, H, 12·7%); X-ray long spacing 39·57 Å, n_D^{70} 1·4320.

Fractions 3–7 obtained by refractionation of A3, 12 and A4, 17, and fractions A4, 18 and 19 were combined and crystallized from methanol (40 vol. at −20°), giving 8·96 g. precipitate. This on fractional distillation yielded: (1) 4·4 g., saponification equiv., 284·4, m.p. of acid 60·0–60·5° undepressed by pure heptadecanoic acid, and another fraction (2) 1·9 g., saponification equiv. 289·7, containing by calculation 1·2 g. methyl heptadecanoate. Crystallization of fraction DI, 7 gave 0·7 g., the acid from which melted at 60·0–60·5° (undepressed by pure heptadecanoic acid).

DISCUSSION

The C and H analyses and saponification equivalents of fractions C3I and of E3I correspond to those required respectively for a C_{15} and a C_{17} saturated acid. The melting point of C3I (52·2–52·7°) agrees with those reported for *n*-pentadecanoic acid, 52·1° (Francis, Piper & Malkin, 1930), 53·0° (Levene & West, 1914), 52·3–53·3° (Links & de Groot, 1953), 54·4° (Coops, quoted by Links & de Groot, 1953), 52·4° (Weitkamp, 1945) and 52·26° (Meyer & Reid, 1933). The melting point of E3I (61·0–61·3°) corresponds with the values recorded for *n*-heptadecanoic acid, 61·3° (Francis & Piper, 1939) and 59·0–60·0° (Levene & West, 1914). The X-ray long spacings of C3I (35·55 Å) and of E3I (39·57 Å) respectively approximate to that reported by Slagle & Ott (1933) for pentadecanoic acid (35·75 Å) and with the values of 40·45 and 40·05 Å for heptadecanoic acid reported respectively by Francis & Piper (1939) and by Slagle & Ott (1933). The refractive indices of fractions C3I and E3I of n_D^{60} 1·4328 and n_D^{70} 1·4320, correspond with those given by Dorinson, McCorkle & Ralston (1942) for pure *n*-pentadecanoic (n_D^{60} 1·4329) and *n*-heptadecanoic (n_D^{70} 1·4324) acids respectively.

Whereas normal-even-numbered fatty acids on solidfication adhered closely to the wall of the flask, the acids isolated in this work, on solidification, moved away from the walls of the flask. Francis *et al.* (1930) have noted that the normal-odd-numbered fatty acids shrink away from the walls of the flask.

From these results it is clear that both *n*-pentadecanoic and *n*-heptadecanoic acids occur in the shark-liver oil examined.

The 10·1 g. of practically pure *n*-pentadecanoic acid isolated in the present work, after correction for losses due to manipulation and withdrawal of samples for analysis, becomes 17·1 g. which, based on the weight of fatty acids taken (6010 g.), is 0·28%. Similarly, the weight of pure *n*-heptadecanoic acid isolated (7·8 g.), together with that calculated on the basis of the equivalents from two slightly impure fractions (1·2 g.), after correction, becomes 10·3 g., which is 0·17% of the total fatty acids.

Hansen *et al.* (1954*a*, *c*) estimated *n*-pentadecanoic and *n*-heptadecanoic acids to be present in hydrogenated mutton fat to the extent of 0·15 and 1·2% respectively, while Shorland *et al.* (1955) calculated that the fatty acids of butterfat contained 0·82% pentadecanoic acid. Thus it would appear that the proportions of these acids in shark-liver oil and ruminant fats are comparable. Whereas in mutton fat heptadecanoic acid was present in greater amounts than pentadecanoic acid, in the shark-liver oil examined the reverse was true.

The origin of the odd-numbered carbon fatty acids in the shark-liver oil studied is at present uncertain, but it is perhaps possible that, as in ruminants, the amino acids of the proteins are broken down to lower fatty acids, including propionic acid, which forms valeric acid in the rumen (Gray, Pilgrim, Rodda & Weller, 1951). By the operation of a similar mechanism to that involved in the building up of even-numbered carbon fatty acids, the long-chain odd-numbered carbon fatty acids could be formed by successive addition of acetate units to propionic acid.

SUMMARY

From the liver oil of the New Zealand school shark (*Galeorhinus australis* Macleay) *n*-pentadecanoic and *n*-heptadecanoic acids have been isolated in amounts corresponding respectively to 0·28 and 0·17% of the total fatty acids.

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Studies in the Biochemistry of Micro-organisms

96. THE COLOURING MATTERS OF *PENICILLIUM HERQUEI* BAINIER & SARTORY*

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One of the four major sections—the Biverticillata–Symmetrica—into which Raper & Thom (1949) divide all species in the genus *Penicillium* was designed by them to include species having, among other characteristics, biverticillate conidial structures which are usually symmetrical. They state (p. 558): 'Penicillia can almost invariably be placed here if they produce... aerial hyphae more or less yellow pigmented and encrusted and colony reverse in yellow, orange or red to purplish red shades.'

Workers in this laboratory have isolated in pure crystalline form the colouring matters produced, by a number of well-defined species of Biverticillata–Symmetrica from three of the six series into which this major section is divided. These colouring matters, although often showing clear signs of chemical inter-relationship, belong to a number of different chemical types. Thus stipitatic acid, $C_8H_6O_5$, from the bright-yellow *P. stipitatum* Thom in the *P. luteum* series (Birkinshaw, Chambers & Raistrick, 1942) is β-hydroxytropolone-β'-carboxylic acid, which is pale yellow in colour. Another bright-yellow species in the same series, *P. wortmanni* Klöcker, afforded two crystalline colouring matters—rugulosin, $C_{30}H_{22}O_{10}$, forming bright-yellow cubes or prisms, and skyrin, $C_{30}H_{18}O_{10}$, reddish orange rods (Breen *et al.* 1955). Seven different pigments were isolated from strains of *P. islandicum* Sopp in the *P. funiculosum* series, colonies of which vary in colour from yellow-orange through orange-red to red. They were: islandicin, bright-red plates, $C_{15}H_{10}O_5$, 1:4:5-trihydroxy-2-methylanthraquinone (Howard & Raistrick, 1949); chrysophanol, golden plates, $C_{15}H_{10}O_4$, 4:5-dihydroxy-2-methylanthraquinone (Howard & Raistrick, 1950); skyrin and flavoskyrin, $C_{15}H_{12}O_5$, light-yellow needles, of unknown structure but converted almost quantitatively into chrysophanol on heating (Howard & Raistrick, 1954*a*); iridoskyrin, deep-red plates, $C_{30}H_{18}O_{10}$; rubroskyrin, dark-red plates, $C_{30}H_{22}O_{12}$; and erythroskyrin, dark-red micro-crystals, and the only pigment in this series containing nitrogen, $C_{24}H_{31}O_6N$ (Howard & Raistrick, 1954*b*). Rubroskyrin is readily converted, by treatment with concentrated sulphuric acid at room temperature, into iridoskyrin and, *inter alia*, into islandicin, by thermal decomposition. Finally, rugulosin and skyrin were also isolated from *P. rugulosum* Thom, in the *P. rugulosum* series, colonies of which have yellow to orange-brown, but seldom a red, reverse (Breen *et al.* 1955). It will

* Part 95: Breen, Dacre, Raistrick & Smith (1955).

thus be seen that skyrin is a colouring matter which is common to some species in each of the three series examined and that rugulosin is common to two series. Neither the molecular structure of skyrin nor that of rugulosin has yet been determined, but it is already clear that they must both be structurally related to some of the other colouring matters described, which are substituted polyhydroxyanthraquinones, since skyrin is smoothly converted, at room temperature by sodium dithionite, into two molecules of emodin (4:5:7-trihydroxy-2-methylanthraquinone) and rugulosin can be converted, also by simple processes though less efficiently, into chrysophanol and emodin.

Work was commenced here in 1950 on the colouring matters of a strain of *Penicillium herquei* Bainier & Sartory, a member of the *P. herquei* series of the Biverticillata–Symmetrica, and two crystalline colouring matters were soon isolated from this strain, which are quite different from any of those mentioned above. While this work was in progress, a preliminary announcement of work on the pigments of *P. herquei* appeared (Stodola, Raper & Fennell, 1951) in which one unnamed pigment was described, having the empirical formula $C_{20}H_{20}O_7$ and melting at 225–227° (decomp.), which was clearly identical with one of the two pigments isolated here. From subsequent correspondence it transpired that Dr Stodola had arranged for further studies on this colouring matter, now named herqueinone, to be carried out by Professor James Cason, Department of Organic Chemistry, University of California, Berkeley, U.S.A. Through the courtesy of Dr Stodola it has been arranged that the American workers' findings and our own should be published simultaneously.

Members of the *P. herquei* series 'are easily recognized, for the bright yellow-green colours that characterize the vegetative mycelium, and usually the colony reverse, are not encountered elsewhere in the genus *Penicillium*' (Raper & Thom, 1949, p. 658). Our strain of *P. herquei*, S.M. 138, was grown on Raulin–Thom solution at 24° in the dark. After 21–28 days' incubation, the yellowish green mycelium, with a bright brownish red reverse, was collected, washed with water and dried *in vacuo* at 40–45°. The dried mycelium was extracted with cold chloroform and the green extract was evaporated *in vacuo* to low bulk, when a considerable quantity of herqueinone, $C_{20}H_{20}O_7$, separated in red needles and was collected. The solvent was removed from the chloroform mother liquors and the dark greenish residue was freed from 'fat' by washing with light petroleum. The resulting dark bluish green amorphous powder remaining contains a complex mixture of colouring matters, only one of which has been isolated in a state of purity. It has been identified as physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, i.e. the 7-methyl ether of emodin.

The chloroform-extracted mycelium was now dried and re-extracted exhaustively with ether, giving good yields of a brick-red crystalline colouring matter, which has not been described previously and for which we propose the name norherqueinone, with the empirical formula $C_{19}H_{18}O_7$. Along with this colouring matter, the ether extract also contained appreciable amounts of *meso*erythritol, $C_4H_{10}O_4$, butane-1:2:3:4-tetraol, colourless tetragonal plates, m.p. 120–124°. This substance, which is of frequent occurrence in algae and lichens, was first described as a mould metabolic product from laboratory cultures of *Penicillium brevi-compactum* Dierckx and *P. cyclopium* Westling (Oxford & Raistrick, 1935).

The total weight of colouring matters which are extractable by organic solvents from the dried mycelium of many of the species of *Penicillium* in the Biverticillata–Symmetrica which we have examined is often very surprisingly large. Thus, with strain N.R.R.L. 1036 of *P. islandicum*, 201 g. of dried mycelium yielded 44·92 g. (≡22·3 %) of crude colouring matter; with strain S.M. 1 of *P. rugulosum*, 200 g. of dried mycelium gave 45·0 g. (≡22·5 %) of crude crystalline rugulosin; and with strain 138 of *P. herquei*, 335 g. of dried mycelium gave a total of 56·45 g. (≡16·8 %) of crude colouring matters.

Herqueinone

Herqueinone, $C_{20}H_{20}O_7$, melts with decomposition, at about 226°. It sublimes in high vacuum at 175–190°. Solutions of herqueinone in ethanol are strongly dextrorotatory. The molecule of herqueinone contains a minimum of three hydroxyl groups, one methoxyl group and at least two methyl groups attached to carbon. It dissolves in aqueous sodium hydroxide, giving a bright-orange solution which rapidly fades to pale yellow, but does not dissolve in sodium carbonate solution. A solution in cold concentrated sulphuric acid is a rather weak orange with a slight yellow fluorescence which is intensified to a strong green on standing for some days. In ethanolic solution it gives a ferric reaction which appears brownish olive by transmitted light but is almost black by reflected light.

The following functional derivatives of herqueinone have been prepared: trimethyl ether A, $C_{23}H_{26}O_7$, pale-yellow rods, m.p. 345°; trimethyl ether B, $C_{23}H_{26}O_7$, colourless rods, m.p. 170–171°. Both the trimethyl ethers are optically inactive and insoluble in aqueous sodium hydroxide, and give negative ferric reactions in ethanol.

Herqueinone is readily converted into an optically inactive isomer, *iso*herqueinone, orange

needles, m.p. 248–249°, by boiling its acetone solution for 1 hr. over solid anhydrous potassium carbonate. Hence it appears probable that both the trimethyl ethers, A and B, are in fact ethers of *iso*herqueinone. *iso*Herqueinone strongly depresses the melting point of herqueinone (226°) on admixture with it, but gives reactions with cold concentrated sulphuric acid, aqueous 2N sodium hydroxide and ferric chloride in ethanol which are indistinguishable from those given by herqueinone with the same reagents.

The infrared absorption spectra of herqueinone and *iso*herqueinone in dilute chloroform solution were identical, within the limits of experimental error, indicating that the change from herqueinone to *iso*herqueinone is a steric change and that no fundamental rearrangement has taken place.

When a solution of herqueinone in glacial acetic acid is treated with zinc dust at room temperature, a reduction product, deoxyherqueinone, $C_{20}H_{20}O_6$, is formed. This substance, which forms yellow plates, m.p. 240–241°, still contains the methoxyl group which is present in the parent substance. It is dextrorotatory, dissolves in aqueous 2N sodium hydroxide giving a pale-yellow solution, but does not dissolve in sodium carbonate. Its reactions with cold concentrated sulphuric acid and ferric chloride in ethanol are almost indistinguishable from those given by the parent substance.

A crystalline product containing bromine is formed when saturated bromine water is added, at room temperature, to an ethanolic solution of herqueinone. This substance, to which the empirical formula $C_8H_8O_4Br_2$ is tentatively assigned, still contains a methoxyl group. It forms colourless needles, m.p. 189–190° (decomp.), which give a moderate purplish brown ferric colour in ethanolic solution.

When a suspension of herqueinone in aqueous 2N sulphuric acid is boiled for some hours, hydrolysis of the herqueinone takes place according to the following equation, only negligible amounts of carbon dioxide being formed:

$$C_{20}H_{20}O_7 + H_2O \rightarrow C_5H_{10}O + C_{15}H_{12}O_7.$$

The substance $C_5H_{10}O$, which was first isolated as the 2:4-dinitrophenylhydrazone, was identified as methyl *iso*propyl ketone. This identification was confirmed by the preparation of its semicarbazone.

The substance $C_{15}H_{12}O_7$ appears not to have been described previously and, because of its colour, we propose to call it xanthoherquein. It forms golden-yellow needles, m.p. 295–296° (decomp.), and the molecule contains one methoxyl group and one methyl group attached to carbon. It is readily soluble in aqueous sodium bicarbonate, giving a yellow solution which changes to orange-red on standing. Its solution in cold concentrated sulphuric acid, initially greenish yellow in colour with a strong yellow-green fluorescence, slowly becomes orange on long standing. It gives a strong olive-green ferric colour in ethanolic solution. The following functional derivatives have been prepared: xanthoherquein perchlorate,

$$C_{15}H_{12}O_7,HClO_4,H_2O,$$

red to orange-brown rectangular plates, m.p. 244° (decomp.); xanthoherquein tetramethyl ether, $C_{19}H_{20}O_7$, bright-yellow needles, m.p. 141–142°, insoluble in aqueous alkalis, but, paradoxically, extractable from ether solution by 2N sodium hydroxide but not from chloroform solution; it gives a brown ferric colour in ethanol; xanthoherquein tetramethyl ether ferrichloride,

$$C_{19}H_{20}O_7,HFeCl_4,$$

orange-brown needles, m.p. 158–159°.

When a solution of herqueinone in aqueous sodium hydroxide is heated in an atmosphere of nitrogen, the breakdown of the colouring matter follows a completely different course from that of the acid hydrolysis. Acetaldehyde is formed in good yield in place of methyl *iso*propyl ketone, but neither xanthoherquein nor any other pure crystalline breakdown product has at present been isolated.

Norherqueinone

The brick-red crystalline material obtained by prolonged ether extraction of the chloroform-extracted mycelium contained a hitherto undescribed colouring matter, which we have named norherqueinone, because, as will be shown later, herqueinone is its monomethyl ether. Norherqueinone, $C_{19}H_{18}O_7$, crystallizes in dark-red needles, m.p. 279° (decomp.), solutions of which in pyridine are very strongly dextrorotatory, $[\alpha]_D + 1080°$. It is almost insoluble in the usual organic solvents. It gives reactions with alkalis and with cold concentrated sulphuric acid which are very similar to those given by herqueinone, but its intense olive-green ferric reaction in ethanol is a much clearer green.

Unlike herqueinone, norherqueinone does not contain a methoxyl group, but the relationship between the two substances is evident from the fact that on methylation it gives a tetramethyl ether, $C_{19}H_{14}O_3(OCH_3)_4$, pale-yellow plates, m.p. 341°, which is identical with the trimethyl ether A of herqueinone.

Like herqueinone, norherqueinone is readily converted into an isomer, *iso*norherqueinone, $C_{19}H_{18}O_7$, red needles, m.p. 284° (decomp.), $[\alpha]_D - 730°$, whereas *iso*herqueinone is optically inactive. *iso*Norherqueinone gives reactions with alkalis, cold concentrated sulphuric acid and ferric

chloride in ethanol which are indistinguishable from those given by the parent substance.

Norherqueinone also undergoes a similar hydrolysis, when boiled with aqueous 2N sulphuric acid, to that of herqueinone with the same reagent. The reaction proceeds, in the same way as with herqueinone, according to the equation

$$C_{19}H_{18}O_7 + H_2O \rightarrow C_5H_{10}O + C_{14}H_{10}O_7,$$

the products of hydrolysis being again one molecule of methyl *iso*propyl ketone and, in this instance, one molecule of norxanthoherquein, $C_{14}H_{10}O_7$, in place of one molecule of xanthoherquein.

Norxanthoherquein, which has not been described previously, forms orange-yellow plates, which do not melt below 360°. The molecule contains one methyl group attached to carbon but, unlike xanthoherquein, does not contain a methoxyl group. Its colour reactions, e.g. formation of a pale-yellow solution in saturated aqueous sodium bicarbonate and a yellow solution with an intense yellow-green fluorescence in cold concentrated sulphuric acid, are very similar to those given by xanthoherquein. In contrast, however, it gives with ethanolic ferric chloride a colour which is initially olive green but which changes to purple brown on standing.

The following functional derivative of norxanthoherquein has been prepared: norxanthoherquein pentamethyl ether, $C_{14}H_5O_2(OCH_3)_5$, yellow needles, m.p. 140–141°, identical with xanthoherquein tetramethyl ether. Xanthoherquein is thus the monomethyl ether of norxanthoherquein.

It is hoped to discuss in a later paper the bearing of the derivatives and breakdown products of herqueinone and norherqueinone on the molecular constitution of these substances.

EXPERIMENTAL

Culture

The strain of *Penicillium herquei* Bainier & Sartory which was used in this investigation was isolated by Mrs S. Marcus in this Department in 1950 from a culture plate exposed at Chelsea Polytechnic, London, by Dr J. C. Dacre. It carries the London School of Hygiene and Tropical Medicine (L.S.H.T.M.) catalogue number S.M. 138. It was identified by Mr G. Smith of this Department.

Cultural characteristics of, and pigment formation by, Penicillium herquei, *strain* S.M. 138

Quantities (350 ml.) of Raulin–Thom solution (glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4,7H_2O$, 0·07 g.; $ZnSO_4,7H_2O$, 0·07 g.; distilled water, 1500 ml.) were distributed in a number of 1 l. conical flasks plugged with cotton wool and sterilized. Batches of 100 such flasks were inoculated with a spore suspension prepared from mature (4–5 week) cultures on beer-wort agar of *P. herquei*, strain S.M. 138, and incubated at 24° in the dark.

The mould grew well on this medium and after 14 days' incubation the cultures developed a strong smell of apples, in agreement with Raper & Thom's (1949, p. 659) observation. After 21 days' incubation the yellowish green mycelium was deeply folded with a bright brownish red reverse. Some areas were covered with a brownish red pigment which, under the microscope, appeared partly amorphous but with many long yellow needles. The culture fluid was moderate brown in colour, with a greenish fluorescence, and contained a number of long orange-yellow needles. A portion of the filtered culture fluid gave a clear olive-brown ferric colour, readily decolorized considerable amounts of saturated bromine water without the formation of a precipitate and, on acidification with HCl, gave a dark-brown amorphous precipitate. Portions of the mycelium, pressed as dry as possible between layers of filter paper, gave the following reactions: with cold conc. H_2SO_4 an immediate very dark brown colour with a strong greenish fluorescence; with aqueous 2N-NaOH or 2N-Na_2CO_3 a deep-brown stable solution.

At the end of the incubation period the mycelium was separated from the culture fluid by filtration, washed well with water, pressed as dry as possible in a tincture press and finally dried in a vacuum oven at 40–45°.

The filtered culture fluid was shown to contain colouring matters, two of which are also present in the mycelium, by the following procedure. The culture filtrate (29·6 l., from ninety-nine flasks of batch 2 after 21 days' incubation, see Table 1) was acidified to Congo red with conc. HCl (75 ml.) and held for 2 days at 0°. The resulting brown amorphous precipitate was separated by filtration, washed and dried (15·5 g.), and part of it (4 g.) was extracted three times with boiling $CHCl_3$ (80, 80, 160 ml.). Evaporation of the combined $CHCl_3$ extracts gave 1·10 g. of red needles, m.p. 220–221°, raised to 227° (decomp.) on recrystallization from ethanol and not depressing the m.p. 226° (decomp.) of a pure specimen of herqueinone. The two specimens also gave identical colour reactions.

The clear acid filtrate from the brown amorphous precipitate (15·5 g.), which gave an olive ferric colour, was extracted twice with ether (½ vol. and ½ vol.). The extracted filtrate, which no longer gave a ferric colour, was discarded. The ether extracts were evaporated to small bulk (about 100 ml.) and held overnight at 0°. The crystalline colouring matter which separated (0·78 g.) was collected, m.p. 228°, depressed to 217° after admixture with herqueinone. The colouring matter was fractionally crystallized from ethanol, giving two fractions, the more soluble of which consisted of herqueinone, m.p. 226° (decomp.) alone or in admixture with an authentic specimen, m.p. 226° (decomp.), isolated from the mycelium. The less soluble fraction (0·45 g.), m.p. about 266°, was repeatedly crystallized from glacial acetic acid (1 l./g.), giving red needles with a metallic sheen (0·13 g.), m.p. 278° (decomp.). A mixture of this substance with norherqueinone isolated from the mycelium, m.p. 279° (decomp.), melted at 279° (decomp.). The two specimens also gave identical reactions in the following tests: (i) in aqueous 2N-NaOH, a dark-red solution fading in a few seconds to pale yellow; (ii) in cold conc. H_2SO_4, an orange-red solution changing on standing at room temperature to reddish brown with a strong green fluorescence; (iii) an intense olive-green ferric colour in ethanol solution.

Isolation of the colouring matters from the mycelium

The vacuum-dried mycelium was immersed for 15–20 hr. in cold $CHCl_3$ (100 ml. for the mycelium of each flask) and the mixture was shaken occasionally. The filtered $CHCl_3$ extract was evaporated *in vacuo* to low bulk (250–300 ml./100 flasks). The dark-green concentrate, which contained much reddish crystalline material, was kept in the refrigerator to complete crystallization and was then filtered. The clean reddish crystalline material obtained was washed with cold $CHCl_3$ and dried, giving almost pure herqueinone (fraction A 1). A second crop (fraction A 2) of only slightly less pure material was obtained on further evaporation of the dark green $CHCl_3$ mother liquors and washings. The yield of herqueinone (fraction A 1 + fraction A 2) obtained was 23 g.–29 g./100 flasks. Full details of the three batches are given in Table 1.

The final $CHCl_3$ mother liquors were evaporated to dryness *in vacuo* and the resulting dark residue was triturated thoroughly with light petroleum (b.p. 40–60°) to remove fatty materials. The mixture was filtered and the undissolved portion was well washed on the filter with light petroleum and then dried, giving fraction B as a dark bluish green powder (see Table 1, column 9). On removal of the solvent from the combined light-petroleum filtrate and washings there remained a residue of crude 'fat', fraction C (Table 1, column 10).

The mycelium, after thorough extraction with $CHCl_3$ as described above, was air-dried and then exhaustively extracted (100 hr.) with ether in a Soxhlet apparatus. When so treated the $CHCl_3$-extracted mycelium (284 g.) of the seventy-seven flasks of batch 1 gave 16·45 g. of crystalline reddish brown colouring matter which separated from the ether extract and purification of which gave *norherqueinone*, m.p. 279° (decomp.). On evaporation of the ether mother liquors there remained a semi-gummy residue (3·1 g.).

The dark bluish green fraction B was shown, by chromatographing its $CHCl_3$ solution on a $MgCO_3$ column, to contain a rather intractable complex mixture of colouring matters which has not yet been fully investigated. However, one of its constituents was shown to be physcion (4:5-dihydroxy-7-methoxy-2-methylanthraquinone) by the following experiment. Fraction B from batch 1 (5 g.) was extracted twice, with 500 ml. each time of light petroleum (b.p. 40–60°), the mixtures being held at room temp. for 24 hr. with occasional shaking. The extract was evaporated to dryness, giving a partly crystalline residue which was washed with a little ether. The undissolved residue was crystallized from $CHCl_3$, giving yellow plates (0·03 g.), m.p. 198–200°, which were purified by sublimation in high vacuum at 125–145°, giving orange needles, m.p. 207–208°. A mixture with authentic physcion, m.p. 206°, from the lichen *Xanthoria parietina* melted at 207°. The two substances also gave identical reactions with the following reagents: cold conc. H_2SO_4, deep-red non-fluorescent solution; aqueous N-NaOH, red solution; aqueous N-Na_2CO_3, insoluble; ethanolic $FeCl_3$, reddish brown colour.

The major portion of the 5 g. of fraction B remained undissolved. It was dried and fractionally extracted in a Soxhlet apparatus with light petroleum (b.p. 40–60°) for 37 hr. The only crystalline material which was obtained was crude herqueinone (small amounts), m.p. 216°. Undissolved material (3·1 g.) remained in the Soxhlet thimble.

Table 1. *Cultural characteristics of* Penicillium herquei *Bainier & Sartory (strain* S.M. 138*) and yields of colouring matters formed by it*

Batch no.	No. of flasks	Incubation period (days)	pH	Glucose by polarimeter (%)	Dried mycelium (g.)	Herqueinone fraction A 1		Herqueinone fraction A 2		Fraction B (g.)	Fraction C crude fat (g.)	Crude crystalline norherqueinone (g.)
						g.	m.p.	g.	m.p.			
1	3	7	2·7	2·24	11·87	0·57	194–198°	None	None	—	—	—
1	10	9	3·4	1·66	42·65	1·58	219–220°	0·34	219–220°	1·1		
1	10	13	3·5	0·80	46·83	1·25	222–223°	0·64	222–223°	1·78	2·93	—
1	77	19	4·2	0·30	335·0	17·62	223–224°	1·4	219–220°	20·98	10·97	16·45
2	99	21	4·6	0·10	444·0	28·71	—	—	—	40·64	8·7	—
3	99	28	—	—	349·0	23·45	—	—	—	34·21	3·08	—

Herqueinone

Most of the preparations of herqueinone which separate directly from the evaporated $CHCl_3$ mycelial extracts melt in the region of 223° and are sufficiently pure for preparative purposes. Herqueinone was purified for analysis in the following way: 3 g. (batch 1; 77 flasks; fraction A 1, m.p. 223–224°) were dissolved in boiling $CHCl_3$ (100 ml.), filtered and cooled to crystallize, giving red crystals (1·58 g.), m.p. 224°, which were recrystallized from ethanol (50 ml.). Herqueinone (0·73 g.) was thus obtained as long red needles, m.p. 226° (decomp.), unchanged on further crystallization. (Found: N, S, Cl, nil; C, 64·2, 64·2; H, 5·4, 5·3; *C*-CH_3, 7·3. Calc. for $C_{20}H_{20}O_7$: C, 64·5; H, 5·4; 2 *C*-CH_3, 8·1%.) Six methoxyl estimations varied from 8·35 to 9·46%, with an average of 8·86%; calc. for 1 OCH_3, 8·34%. Stodola *et al.* (1951) describe similar results. $[\alpha]_D^{22} + 440° \pm 40°$; $[\alpha]_{5461}^{22} + 640° \pm 20°$, in ethanol (*c*, 0·063). Owing to the relatively slight solubility of herqueinone and its derivatives in organic solvents and the intense colour of the solutions obtained, the determination of their optical rotations is difficult and is subject to a large error.

The ultraviolet absorption spectrum of herqueinone in ethanol solution showed the following maxima: $\lambda_{max.}$ 220, 250, 314, 416 mμ. (log ε 4·29, 4·09, 4·47, 3·66 respectively).

Herqueinone crystallizes in brick-red needles from $CHCl_3$, ether, ethanol and ethyl acetate, being fairly soluble at room temperature in all four solvents, e.g. 1 g. dissolves in 175 ml. of ethanol. It is insoluble in light petroleum and water. It sublimes in high vacuum at 175–190° in crystalline form but with slight decomposition.

Herqueinone dissolves in 2N-NaOH, giving a deep-orange solution fading to pale yellow in less than 1 min., but is not soluble in aqueous Na_2CO_3. It also dissolves readily in conc. HCl, giving a reddish orange solution, and in cold conc. H_2SO_4, giving a rather weak orange solution with a slight yellow fluorescence. On standing, the solution darkens in colour and takes on a strong green fluorescence. A saturated ethanolic solution of herqueinone which is not acid to litmus gave the following reactions: with ethanolic $FeCl_3$, an intense black colour which appears as a brownish olive colour in very dilute solution; with saturated bromine water, decolorization and the separation of a colourless crystalline product; with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl), the formation of red needles of unchanged herqueinone. A solution of herquienone in acetic acid, initially red in colour, quickly becomes orange yellow on the addition of Zn dust.

iso*Herqueinone*. In an unsuccessful attempt to methylate herqueinone with methyl iodide, a mixture of herqueinone (1 g.), methyl iodide (5 g.), anhydrous K_2CO_3 (5 g.) and dry acetone (100 ml.) was refluxed for 8 hr. and was then filtered while still hot. The acetone filtrate, which still gave a strong ferric colour, was evaporated to dryness *in vacuo* and the residue was extracted with water and filtered. The aqueous filtrate was acidified and extracted with ether. The ethereal extract was evaporated, giving orange needles (0·27 g.), m.p. 238°. This product was crystallized thrice from ethanol and gave iso*herqueinone* as orange needles, m.p. 248–249°, and strongly depressing the m.p. of herqueinone (226°) on admixture with it. (Found: C, 64·3; H, 5·4; OCH_3, 7·9. $C_{20}H_{20}O_7$ requires C, 64·5; H, 5·4; 1 OCH_3, 8·3%.) The substance does not dissolve in N-Na_2CO_3, but dissolves in aqueous 2N-NaOH, giving a deep-orange solution, which fades to pale yellow in less than 1 min. It gives reactions with cold conc. H_2SO_4 and $FeCl_3$ in ethanol which are indistinguishable from those given by herqueinone with the same reagents. It is optically inactive, $[\alpha]_D^{20}$ nil, in ethanol (*c*, 0·045). *iso*Herqueinone was also formed when the process was repeated, (*a*) using dry benzene (200 ml.) in place of acetone (100 ml.) and (*b*) omitting the methyl iodide and heating the herqueinone–K_2CO_3–acetone mixture for only 1 hr.

The infrared absorption spectra of herqueinone and *iso*herqueinone were kindly determined for us by Dr K. T. Potts, of the National Institute for Medical Research, Mill Hill. The spectra were measured in $CHCl_3$ solution (0·5%) in a 1 mm. cell on a Perkin Elmer Model 21 spectrophotometer with a rock-salt prism. The spectra of the two substances were identical within the limits of experimental error and showed the following main absorption bands: 3640 m, 3570 s, 3300 m, 2980 s, 1625 s, cm.$^{-1}$ (s = strong, m = medium absorption).

Deoxyherqueinone. Zn dust (2 g.) was added in small portions during 15 min. to a solution of herqueinone (1 g.) in glacial acetic acid (150 ml.) at room temp. The mixture was filtered and the orange-yellow non-fluorescent filtrate was poured into water (300 ml.), giving a yellow precipitate which was collected, thoroughly extracted with aqueous N-HCl (50 ml.), washed with water and dried (0·77 g.); m.p. 235–237°. The dry yellow powder was repeatedly crystallized from benzene, giving *deoxyherqueinone* as yellow plates, m.p. 240–241°. (Found, on sample dried at 100° in high vacuum with no loss in weight: C, 67·3; H, 5·6; OCH_3, 8·4, 8·85. $C_{20}H_{20}O_6$ requires C, 67·4; H, 5·7; 1 OCH_3, 8·7%.) $[\alpha]_D^{22} + 64°$; $[\alpha]_{5461}^{22} + 68°$ in acetic acid (*c*, 0·50). It is soluble in aqueous 2N-NaOH, giving a pale-yellow solution, but does not dissolve in Na_2CO_3. It dissolves in cold conc. H_2SO_4 to an immediate bright-yellow solution with an intense yellowish green fluorescence. It gives in ethanol an intense brownish olive ferric colour which is indistinguishable from that given by herqueinone tested alongside.

Methylation of herqueinone with dimethyl sulphate. A mixture of herqueinone (2 g.), dry acetone (200 ml.), dimethyl sulphate (20 ml.) and anhydrous K_2CO_3 (40 g.) was boiled under a reflux condenser, fitted with a $CaCl_2$ guard tube, for 2½ hr., when the clear supernatant, initially red, had become pale-yellow in colour and ceased to give a ferric reaction.

The mixture, while still hot, was filtered on a Büchner funnel. The residue in the funnel was washed with acetone and then with water (500 ml.). The undissolved portion (0·30 g.) was combined with similar material (0·115 g.) from the acetone filtrate (see below) and was purified by crystallization from glacial acetic acid (225 ml.) and sublimation (no residue) in high vacuum at 270–295°. *Trimethylherqueinone A* was thus obtained as pale-yellow rods, m.p. 345° with the formation of a sublimate, which itself resublimes at 325–330°. (Found: C, 66·55; H, 6·7; OCH_3, 30·1. $C_{23}H_{26}O_7$ requires C, 66·7; H, 6·3; 4 OCH_3, one of which is present in herqueinone, 29·95%.) Trimethylherqueinone A is optically inactive, $[\alpha]_D^{20}$ nil, in benzene (*c*, 0·25). It is insoluble in 2N-NaOH and gives no ferric colour in ethanolic solution.

The acetone filtrate and washings were evaporated *in vacuo*, and the yellow crystals of trimethylherqueinone A (0·115 g., see above), which separated from the viscous residue, were collected by filtration. The resulting viscous filtrate was dissolved in ether (100 ml.), and the ethereal solution was extracted with aqueous 2N-NaOH (3 × 50 ml.)

and then with water (2 × 50 ml.) and then evaporated to dryness. The viscous residue was stirred vigorously with aqueous N-NaOH (300 ml.) and set aside overnight at 0°. The solid which separated was collected, washed with water and dried (1·14 g.). It was dissolved in ether and crystallized, by slow evaporation of the solvent, in colourless prisms (0·19 g.), m.p. 163–167°. Purification of this substance by crystallization from ether–light petroleum, b.p. 40–60°, and sublimation in high vacuum at 150–170° gave *trimethylherqueinone* B as colourless rods (0·13 g.), m.p. 170–171°. (Found: C, 66·95; H, 6·3; OCH_3, 30·0. $C_{23}H_{26}O_7$ requires C, 66·7; H, 6·3; 4 OCH_3, one of which is present in herqueinone, 29·95%.) Trimethylherqueinone B is optically inactive, $[\alpha]_D^{20}$ nil, in benzene (*c*, 0·25). It is insoluble in 2N-NaOH and gives no ferric colour in ethanolic solution.

The only crystalline product isolated from methylation of herqueinone (1 g.) in acetone (80 ml.) for 3 hr. with much smaller amounts of dimethyl sulphate (0·9 ml.) and K_2CO_3 (2 g.) was the yellow trimethyl ether A, m.p. 341°, which did not depress the m.p. of the specimen described above, m.p. 345°.

Bromine derivative from herqueinone. Saturated bromine water (500 ml.) was added to a solution of herqueinone (2 g.) in ethanol (350 ml.), and the mixture was held at 0° overnight. The colourless crystalline material which separated was collected and dried (0·95 g.), m.p.175–176°. By the further addition of bromine water (250 ml.) and water (500 ml.) to the filtrate, a second crop (1·27 g.), m.p. 105–112°, of colourless amorphous material, which could not be crystallized, was obtained. The crystalline material, m.p. 175–176°, was crystallized from $CHCl_3$–ethanol and $CHCl_3$–ether, and the *bromine derivative* was obtained as colourless needles, m.p. 189–190° (decomp.). (Found: C, 29·1; H, 2·6; Br, 48·6; OCH_3, 8·2. $(C_8H_4O_2Br)_n$ requires C 29·3; H, 2·5; Br, 48·7; 1 OCH_3 if $n=2$, 9·5; 1 OCH_3 if $n=3$, 6·3%.) The substance does not dissolve in aqueous $NaHCO_3$, but dissolves slowly in N-NaOH. It is readily soluble in $CHCl_3$, much less soluble in ethanol and almost insoluble in water. Its saturated ethanolic solution gives a moderate purplish brown ferric colour.

Acid hydrolysis of herqueinone

A slow stream of CO_2- and O_2-free N_2 was bubbled through a suspension of herqueinone (1·0 g.) in aqueous 2N-H_2SO_4 (150 ml.) contained in a flask fitted with a tap funnel and a short upright condenser. The effluent gases were first passed through a number of bubblers containing Brady's reagent and then through further bubblers containing standard aqueous $Ba(OH)_2$. The contents of the flask were boiled gently and, soon after boiling commenced, a yellow precipitate, A, began to appear in the Brady bubblers. Boiling was continued for 13–14 hr., after which time only a negligible amount of CO_2 had been absorbed in the baryta bubblers. After about 9 hr. boiling, the cooling water was drained from the reflux condenser to facilitate the removal of volatile products from the reaction mixture, and water was slowly added through the tap funnel to replace the distillate. At the end of the hydrolysis, the contents of the reaction flask (hydrolysis product B) were held at 0° overnight.

Treatment of yellow precipitate A. Isolation of the 2:4-*dinitrophenylhydrazone of methyl* iso*propyl ketone.* The yellow precpitate A in the Brady bubblers was collected by filtration, washed with water and dried (0·292 g.), m.p. 117°. It was purified by repeated crystallization from ethanol and was finally obtained as yellow needles, m.p. 122–123°. (Found: C, 49·8; H, 5·0; N, 21·0. $C_{11}H_{14}O_4N_4$, i.e. the 2:4-dinitrophenylhydrazone of $C_5H_{10}O$, requires C, 49·6; H, 5·3; N, 21·05%.)

The melting point was not depressed by admixture with an authentic sample of the 2:4-dinitrophenylhydrazone of methyl *iso*propyl ketone. The distillate from an acid hydrolysis of herqueinone was extracted with ether. The residue left after careful removal of the ether was treated with semicarbazide hydrochloride. The product, after recrystallization from light petroleum (b.p. 60–80°), was methyl *iso*propyl ketone semicarbazone, m.p. 113–114°, unchanged on admixture with an authentic specimen. (Found: C, 50·75; H, 9·0; N, 29·6. Calc. for $C_6H_{13}ON_3$: C, 50·3; H, 9·15; N, 29·3%.)

The formation of methyl *iso*propyl ketone on acid hydrolysis of herqueinone suggests the probable origin of the high methoxyl values found for herqueinone, i.e. from 8·35 to 9·46% (calc. for 1 OCH_3, 8·34%), since methyl *iso*propyl ketone gave 0·7% OCH_3 in a Zeisel estimation.

Treatment of hydrolysis product B. Isolation of xantho-herquein. The contents of the reaction flask (hydrolysis product B) were filtered, washed with water and dried (0·70 g.), m.p. 290–293° after sintering at 250–260°. This yellowish brown partially crystalline solid was repeatedly crystallized from aqueous methanol (60:40) and aqueous ethanol (50:50), giving finally golden-yellow needles, m.p. 295–296° (decomp.). (Found, on different samples dried to constant weight in high vacuum at 120–125°: C, 59·3, 59·55; H, 4·1, 4·05; OCH_3, 10·1, 10·1; *C*-CH_3, 4·7. $C_{15}H_{12}O_7$ requires C, 59·2; H, 4·0; 1 OCH_3, 10·2; 1 *C*-CH_3, 4·9%.)

The course of the acid hydrolysis of herqueinone thus proceeds according to the equation

$$C_{20}H_{20}O_7 + H_2O \rightarrow C_5H_{10}O + C_{15}H_{12}O_7,$$

and we propose to name the $C_{15}H_{12}O_7$ hydrolysis product *xanthoherquein* because of its colour.

Xanthoherquein is acid to litmus and is readily soluble in aqueous $NaHCO_3$, giving a yellow solution which changes to orange-red on standing for several hours. Its solution in aqueous 2N-NaOH, initially red in colour, quickly changes to orange-red and, on standing overnight, to brownish red. It dissolves in cold conc. H_2SO_4, giving a greenish yellow solution, slowly becoming orange, and with a strong green fluorescence which fades on long standing. It gives a strong olive-green ferric reaction in ethanolic solution. Its ultraviolet spectrum in ethanol solution showed the following maxima: light-absorption maxima λ 222, 370, 387, ~262, ~277 mμ. (log ϵ, 4·46, 4·19, 4·23, 4·20, 3·98 respectively; ~ = point of inflexion).

Xanthoherquein perchlorate. A few drops of 60% perchloric acid were added to a suspension of xanthoherquein (0·20 g.) in hot glacial acetic acid (10 ml.), when the substance immediately dissolved, forming an orange-yellow solution. Additional perchloric acid (1 ml.) was now added and, on cooling, *xanthoherquein perchlorate* crystallized. It was recrystallized from acetic acid–perchloric acid as rosettes of dark-red rectangular plates, which on drying *in vacuo* at 100° changed colour to orange-brown (0·16 g.), m.p. 244° (decomp.). (Found: C, 42·9; H, 3·5; OCH_3, 7·1; Cl, 8·5. $C_{15}H_{12}O_7,HClO_4,H_2O$ requires C, 42·6; H, 3·6; 1 OCH_3, 7·3; Cl, 8·4%.)

Xanthoherquein tetramethyl ether. A mixture of xanthoherquein (0·10 g.), anhydrous K_2CO_3 (2 g.), dimethyl sulphate (1 ml.) and dry acetone (10 ml.) was boiled under reflux for 1 hr. Acetone was then removed by evaporation, and water (20 ml.) was added to the residue and vigorously shaken. The undissolved dark-yellow residue was collected (0·1 g.), washed with water, dried and crystallized to constant m.p. from methanol, giving *xanthoherquein tetramethyl ether* as bright-yellow needles, m.p. 141–142°. (Found, on material dried in high vacuum at 95° without loss in weight: C, 63·4; H, 5·7; OCH_3, 42·95, 42·6; *C*-CH_3, 4·9. $C_{19}H_{20}O_7$ requires C, 63·3; H, 5·6; 5 OCH_3, 43·05; 1 *C*-CH_3, 4·2%.) Its solution in cold conc. H_2SO_4 is bright golden-yellow in colour without fluorescence. It gives a colour with ethanolic $FeCl_3$ which is brown by transmitted light and olive brown by reflected light. It is insoluble in aqueous $NaHCO_3$, Na_2CO_3 or 2N-NaOH. However, when its solution in ether is shaken with aqueous 2N-NaOH, the substance is slowly extracted into the aqueous alkali, from which, on acidification, it is precipitated (bright-yellow needles from methanol, m.p. 141–142°). On the contrary, it is not extracted from $CHCl_3$ solution by aqueous 2N-NaOH.

Ferrichloride of xanthoherquein tetramethyl ether. The methyl ether (70 mg.) was dissolved in glacial acetic acid (3 ml.), and a solution of $FeCl_3$ (70 mg.) in conc. HCl (5 ml.) was added. The resulting crystals were collected and recrystallized from glacial acetic acid–conc. HCl containing a little $FeCl_3$ and the *ferrichloride of xanthoherquein tetramethyl ether* was obtained as orange-brown needles (55 mg.), m.p. 158–159° (decomp.). (Found: C, 41·1; H, 4·1; Cl, 24·85; Fe, 10·1; OCH_3, 27·3. $C_{19}H_{20}O_7$,$HFeCl_4$ requires C, 40·8; H, 3·8; Cl, 25·4; Fe, 10·0; 5 OCH_3, 27·8%.)

Alkaline hydrolysis of herqueinone

A solution of herqueinone (0·5 g.) in aqueous 2N-NaOH (40 ml.) was boiled in a stream of purified N_2 in the same apparatus as was used for the acid hydrolysis of herqueinone. A yellow precipitate appeared in the Brady bubblers soon after boiling commenced. Precipitation appeared to be complete after 0·5 hr. and the hydrolysis was terminated after 1 hr. The yellow precipitate was collected (97 mg.), m.p. 141–143°, and purified by crystallization from methanol, giving acetaldehyde 2:4-dinitrophenylhydrazone as golden plates, m.p. 164·5–166°, not depressed on admixture with an authentic specimen. (Found: C, 42·95; H, 3·6. Calc. for $C_8H_8O_4N_4$: C, 42·9; H, 3·6%.) Acidification of the alkaline hydrolysate produced a copious precipitate from which, however, no crystalline substance could be isolated.

It thus appears that alkaline hydrolysis of herqueinone follows a different course from that of the acid hydrolysis.

Norherqueinone

The crystalline brick-red colouring matter (16·45 g.; batch 1, 77 flasks), obtained by ether extraction of the $CHCl_3$-extracted mycelium, was fractionated by boiling portions of it for 10 min. with ethanol (250 ml./g.). The mixture was filtered hot and the undissolved portion (0·27 g./2 g.), m.p. 279° (decomp.), was recrystallized from glacial acetic acid (1 l./g.), giving norherqueinone in small red needles, m.p. 279° (decomp.). (Found: N, S, Cl, nil; C, 64·0, 63·8, 63·7, 63·6; H, 5·0, 5·3, 5·0, 5·0; *C*-CH_3, 10·35; OCH_3, 0·80. $C_{18}H_{18}O_7$ requires C, 63·7; H, 5·1; 3 *C*-CH_3, 12·5; 2 *C*-CH_3, 8·4%.) $[\alpha]_D^{23} + 1080° \pm 60°$; $[\alpha]_{5461}^{23} + 1120° \pm 60°$ in pyridine (*c*, 0·048). Norherqueinone is only slightly soluble in most of the usual organic solvents. It dissolves in aqueous 2N-NaOH, giving a dark-red solution which rapidly fades to yellow. It is insoluble in aqueous N-Na_2CO_3, and cannot be extracted by this reagent from its $CHCl_3$ or *n*-butanol solution. Its solution in cold conc. H_2SO_4, initially reddish orange in colour, changes on standing to reddish brown with a strong green fluorescence. It gives an intense olive-green ferric colour in ethanolic solution. The crystals of norherqueinone are light-sensitive, changing on exposure to daylight to a dark-brown colour on the surface.

The hot ethanolic filtrate obtained above by extraction of the crude crystalline colouring matter (250 ml./g.) deposited a small amount of norherqueinone on cooling, and this was collected by filtration. The filtrate was evaporated *in vacuo* to a small volume and a pale-yellow solid separated on standing. This crystallized from methanol as stout, colourless, tetragonal prisms, m.p. 120–124°, undepressed on admixture with authentic *meso*erythritol. Yield, 80 mg. from 1·2 g. of crude colouring matter. (Found: C, 39·3; H, 8·2. Calc. for $C_4H_{10}O_4$: C, 39·3; H, 8·25%.) The substance is very soluble in water, gives no colour with 2N-NaOH, conc. H_2SO_4 or $FeCl_3$ and does not reduce Fehling's solution.

iso*Norherqueinone.* A mixture of norherqueinone (0·5 g.), anhydrous K_2CO_3 (1 g.) and dry acetone (300 ml.) was refluxed for 1 hr. The norherqueinone soon dissolved, giving a brown-red solution which was filtered while still hot. The filtrate was evaporated to dryness and the residual dark red-brown solid was dissolved in a little cold water and acidified with HCl. The resulting bright-red crystalline solid, on recrystallization from acetone or methanol, gave iso*norherqueinone* (0·20 g.) as dark-red needles, m.p. 284° (decomp.). (Found: C, 63·9; H, 4·9. $C_{18}H_{18}O_7$ requires C, 63·7; H, 5·1%.) *iso*Norherqueinone gives colour reactions with 2N-NaOH, cold conc. H_2SO_4 and $FeCl_3$ in ethanol which are indistinguishable from those given by norherqueinone with the same reagents. $[\alpha]_D^{22} - 730° \pm 60°$, $[\alpha]_{5461}^{22} - 790° \pm 60°$ in pyridine (*c*, 0·048).

Methylation of norherqueinone with dimethyl sulphate. A mixture of norherqueinone (2 g.), anhydrous K_2CO_3 (6 g.), dimethyl sulphate (2·8 ml.) and acetone (200 ml.) was refluxed for 18 hr. The acetone solution, initially dark red but now pale yellow in colour, was separated from insoluble material by filtration. The filtrate was evaporated to dryness and the residue was triturated with ether and filtered. No crystalline material could be isolated, either by fractionation with 2N-NaOH or by chromatography on alumina in benzene, from the ether filtrate, which contained much gummy material (cf. methylation of herqueinone). The pale-yellow solid (0·174 g.; m.p. about 330°) remaining undissolved in the ether was purified by crystallization from benzene–light petroleum (b.p. 60–80°), followed by sublimation in high vacuum at 270°, and a final crystallization from glacial acetic acid gave norherqueinone tetramethyl ether as pale-yellow plates, m.p. 341°, which did not depress the m.p. of trimethylherqueinone A, m.p. 345°.

Acid hydrolysis of norherqueinone

A suspension of norherqueinone (1 g.) in 2N-H_2SO_4 (150 ml.) was boiled in N_2 for 96 hr. in the same apparatus and under similar conditions to those used in the acid

hydrolysis of herqueinone. Only a negligible amount of CO_2 was evolved. The yellow precipitate in the Brady bubblers (0·402 g. ≡ 86 % of the theoretical; m.p. 118–119°) was crystallized from methanol, giving orange needles, m.p. 122–123°, not depressed on admixture with authentic methyl *iso*propyl ketone 2:4-dinitrophenylhydrazone.

The solid in the hydrolysis flask, initially dark red but now reddish yellow in colour, was collected, washed and dried (0·88 g.). It was purified by repeated sublimation in high vacuum at about 250°, giving *norxanthoherquein* as yellow-orange plates, which darken slowly on heating above 300° and do not melt below 360°. (Found: C, 58·2, 58·2; H, 3·6, 3·5; OCH_3, 0·3; *C*-CH_3, 5·6. $C_{14}H_{10}O_7$ requires C, 57·9; H, 3·5; 1 OCH_3, 10·7; 1 *C*-CH_3, 5·2 %.) Norxanthoherquein is only very slightly soluble in all the usual organic solvents. It dissolves in saturated aqueous $NaHCO_3$ and in N-Na_2CO_3, forming a pale-yellow solution, which rapidly darkens in colour, and in aqueous 2N-NaOH, giving an orange solution. In cold conc. H_2SO_4 it gives a yellow solution with an intense yellow-green fluorescence. It gives an olive-green ferric colour in ethanol, changing to purple-brown on standing.

Norxanthoherquein can be prepared more expeditiously as follows: A mixture of norherqueinone (1 g.), water (60 ml.), glacial acetic acid (30 ml.) and conc. H_2SO_4 (30 ml.) was refluxed for 16 hr. The norherqueinone dissolved immediately on heating to a dark-red solution, which had changed to yellow-brown at the end of the hydrolysis. The mixture was then partly concentrated *in vacuo*, and the concentrate was poured into water (600 ml.) to which sodium acetate (20 g.) was added. The resulting flocculent brown precipitate was filtered off, washed well with water and dried. The crude norxanthoherquein (0·84 g.) was purified by sublimation as described above. The distillate from the vacuum concentration of the hydrolysis mixture was neutralized with NaOH and extracted with ether. On removal of the solvent from the ether extract, methyl *iso*propyl ketone remained as a fragrant colourless liquid.

Methylation of norxanthoherquein. An etheral solution of diazomethane (from 10 g. of nitrosomethylurea) was slowly added to a suspension, cooled in ice, of norxanthoherquein (1 g.) in methanol (20 ml.). A vigorous evolution of gas occurred and the solid dissolved. The solution was kept at 0° for 24 hr. A further similar amount of diazomethane was then added and the solution was again kept at 0° for 24 hr. Excess of diazomethane was destroyed by addition of acetic acid. The mixture was then washed with water and finally extracted with aqueous 2N-NaOH. On acidification of the alkaline extract, a yellow crystalline solid (0·16 g.) was precipitated and collected. It was recrystallized from methanol, giving pale-yellow needles, m.p. 140–141°, not depressed on admixture with xanthoherquein tetramethyl ether, m.p. 141–142°, and giving identical colour reactions. (Found: C, 63·35; H, 5·7; OCH_3, 42·7. Calc. for $C_{19}H_{20}O_7$: C, 63·3; H, 5·6; 5 OCH_3, 43·05 %.) The alkali-extracted ethereal solution was dried ($MgSO_4$) and evaporated, giving a brown oil which has not been obtained in crystalline form.

SUMMARY

1. The dried mycelium of laboratory cultures of a typical strain of *Penicillium herquei* Bainier & Sartory has yielded, by solvent extraction, about 17 % of its weight of a complex mixture of crude colouring matters.

2. Minor constituents of this mixture are physcion (4:5-dihydroxy-7-methoxy-2-methylanthraquinone) and the colourless tetrahydric alcohol, *meso*erythritol.

3. Two of the major constituents are the hitherto undescribed norherqueinone, $C_{19}H_{18}O_7$, dark-red needles, m.p. 279°, and its monomethyl ether, herqueinone, $C_{20}H_{20}O_7$, brick-red needles, m.p. 226°. Both substances are strongly dextrorotatory.

4. A number of functional derivatives of herqueinone and norherqueinone are described.

5. Acid hydrolysis of herqueinone gives one molecule each of methyl *iso*propyl ketone, $C_5H_{10}O$, and xanthoherquein, $C_{15}H_{12}O_7$, golden-yellow needles, m.p. 295–296°. Similar hydrolysis of norherqueinone gives the same ketone and norxanthoherquein, $C_{14}H_{10}O_7$, orange-yellow plates, m.p. above 360°.

6. Norxanthoherquein and xanthoherquein give the same pentamethyl ether, $C_{14}H_5O_2(OCH_3)_5$. Hence xanthoherquein is the monomethyl ether of norxanthoherquein.

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Studies Involving Enzymic Phosphorylation

5. THE 'ACTIVATION' OF RAT-BRAIN HEXOKINASE BY ERYTHROCYTE LYSATES AND MUSCLE EXTRACTS*

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Weil-Malherbe & Bone (1951*b*, *c*) reported that the hexokinase activity of an aqueous rat-brain extract could be substantially increased by the addition of a human-erythrocyte lysate or rabbit skeletal muscle extract, neither of which itself possessed any appreciable hexokinase activity. Although the 'activator' was non-dialysable and behaved as a protein, they considered that it could not be identified with 6-phosphofructokinase, myokinase or glyceraldehyde 3-phosphate dehydrogenase.

With glucose as substrate, the product of enzymic phosphorylation, glucose 6-phosphate, is an inhibitor of rat-brain hexokinase (Weil-Malherbe & Bone, 1951*a*); it is clear, therefore, that the activator could exert its effect by removing glucose 6-phosphate from the reaction medium. We have repeated the activation experiments of Weil-Malherbe & Bone (1951*b*, *c*), using the specific enzymic methods of Slater (1953) for determining spectrophotometrically the amounts of hexose mono- and di-phosphates formed from glucose in this system. Furthermore, we have carried out parallel experiments with 2-deoxy-D-glucose as substrate. These studies have led us to the conclusion that the major source of activation with glucose as substrate is attributable to the 6-phosphofructokinase that is present in the erythrocyte lysates and muscle extracts. In addition, evidence from other types of experiment has implicated two weaker activating effects, one exerted directly on the hexokinase and the other on the 6-phosphofructokinase present in the rat-brain extracts.

EXPERIMENTAL

Materials

Adenosine triphosphate (*ATP*) was isolated as the barium salt by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948) with the following modification. The whole skinned, eviscerated and decapitated rabbit carcass was used instead of the separated muscle, whereby an increased yield of ATP was obtained. The barium ATP was converted into the potassium salt, a 5% excess of the calculated amount of K_2SO_4 being used, and was stored as a frozen 0·05M solution at −15°.

* Part 4: Long (1955).

Reduced diphosphopyridine nucleotide (*DPNH*) was obtained from C. F. Boehringer and Soehne (Mannheim, Germany).

Hexose monophosphate (*HMP*), a mixture of glucose 6-phosphate (70%) and fructose 6-phosphate (30%), was prepared by the method of Dubois & Potter (1943). The potassium salt was stored as a 0·07M solution at −15°.

2-Deoxy-D-glucose was a gift from Professor M. Stacey, University of Birmingham. In butanol–acetic acid–water (4:1:5, by vol.) it migrated as a single substance on a paper chromatogram, with R_F 0·35 (cf. Edward & Waldron, 1952).

Serum albumin was a sample of 'Albumen (blood)' obtained from British Drug Houses Ltd.

All other substances were the best available commercial preparations. Glass-distilled water was used throughout.

Preparation of extracts containing enzymes and activators

Rat-brain extract. Adult hooded rats of both sexes were used. Aqueous brain extracts were prepared as described by Weil-Malherbe & Bone (1951*a*), and were used either immediately or after storage in the frozen state at −15° for not longer than 4 hr. From the whole brain of one rat, about 9 ml. of extract were obtained.

Yeast hexokinase was prepared according to Slater (1953), after autolysis by the method of Allfrey & King (1950), and was stored at −15° as a paste in 0·75 saturated $(NH_4)_2SO_4$ containing 1% (w/v) glucose. Its high activity was retained for at least 2 years.

Rabbit-muscle fraction A, containing aldolase, triose phosphate isomerase and L-α-glycerophosphate dehydrogenase, was prepared by the procedure of Racker (1947), with the following modification. In order to destroy any contaminating 6-phosphofructokinase (6-PFK) activity, the product was acidified to pH 6 with 0·1N-HCl and dialysed against several changes of glass-distilled water at 0° for 2–3 days. The fraction that was precipitated between 0·5 and 0·75 saturation with $(NH_4)_2SO_4$ was then separated and stored at −15°. It retained its activity for at least 6 months.

Rabbit-muscle fraction B, containing hexose phosphate isomerase and 6-PFK, was prepared according to Slater (1953). Its activity was stabilized by carrying out the isolation in 0·001M ethylenediaminetetraacetate (EDTA). Preparations of high 6-PFK activity, obtained in this way and dissolved in 0·01M ammonium phosphate buffer, pH 7·6, +0·001M EDTA, remained stable on storage at −15° for 6 months.

Erythrocyte lysate. This was prepared from human blood by the method of Weil-Malherbe & Bone (1951*b*) and remained active for several weeks at −15°.

Rat-muscle extract was prepared according to Weil-Malherbe & Bone (1951*c*) and remained active for one week at −15°. Any hexokinase activity could be destroyed completely by heating at 55° for 5 min. and removing the coagulated protein.

Methods

Determination of hexose utilization. Unless otherwise stated, the conditions were similar to those used by Weil-Malherbe & Bone (1951*a*), except that in place of $NaHCO_3$ the system was buffered at pH 7·6 with aminotrishydroxymethylmethane (tris) (Gomori, 1946) or potassium phosphate. The reaction mixture normally contained (final concn. in brackets): glucose or 2-deoxyglucose (0·004 M), ATP (0·005 M), $MgCl_2$ (0·0067 M), KF (0·024 M) and tris or phosphate buffer, pH 7·6 (0·025 M). Rat-brain extract or a diluted solution of the stock yeast-hexokinase preparation (0·05 ml.), with or without activator (0·05–0·15 ml.), was added; total vol., 0·5 ml. After incubation for 20 min. at 30°, reaction mixtures were deproteinized with the $Ba(OH)_2$–$ZnSO_4$ reagents of Somogyi (1945) and glucose was determined in the filtrates by the copper-reduction method of Nelson (1944), according to the procedure described by Long (1951). When 2-deoxyglucose was used as substrate, copper reduction was measured with 'reagent 60' of Shaffer & Somogyi (1933), as recommended by Sols & Crane (1954). When HMP or fructose 1:6-diphosphate (HDP) was to be determined, as described below, reaction mixtures were deproteinized by addition of 0·1 ml. 30% (w/v) trichloroacetic acid (TCA).

Determination of 6-PFK activity. The conditions were identical with those described for hexose utilization, except that potassium HMP (final concn. 0·014 M) replaced hexose, and that the ATP concn. was increased to 0·01 M. Enzyme activity was stopped by adding 0·1 ml. 30% (w/v) TCA. After diluting to 5 ml. with 5% (w/v) TCA and filtering, a known volume of the neutralized filtrate was analysed for HMP and HDP.

Determination of HMP and HDP. By the use of the appropriate rabbit-muscle fractions, HMP and HDP were converted into dihydroxyacetone phosphate, which oxidizes DPNH in the presence of L-α-glycerophosphate dehydrogenase. The spectrophotometric method of Slater (1953) was used, with two modifications: (i) tris buffer (pH 7·6; final concn., 0·025 M) replaced glycylglycine; (ii) it was found necessary to add the ATP and Mg^{2+} to the spectrophotometer cells at the start of the analysis instead of with rabbit-muscle fraction *B*, as otherwise HMP could not be satisfactorily determined. It should be noted that HMP, as determined by this method, includes glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate, while part of the measured HDP may consist of triose phosphate.

RESULTS

Enzymic phosphorylation of glucose and 2-deoxyglucose in the absence of activators

Rat-brain extract. Weil-Malherbe & Bone (1951*a–c*) buffered their reaction mixtures with 0·02 M-$NaHCO_3$ in an atmosphere of N_2+CO_2 (95:5). We have found, however, that the nature of the buffer exerts a marked effect on the rate of glucose utilization by a rat-brain extract (Table 1); the rate with bicarbonate buffer was intermediate between those obtained with tris and phosphate.

When a rat-brain extract was incubated with glucose and ATP in a medium buffered with tris (Fig. 1), the following results were obtained. After 4 min., the glucose disappearing was quantitatively converted into HMP; after 8 min., there was little further increase in HMP concentration and some HDP was formed. As incubation continued, the rate of glucose utilization decreased considerably, formation of HDP was almost linear and there was a fall in HMP concentration. This result is consistent with the presence of hexokinase, phosphohexose isomerase and 6-PFK in the brain extract. After 1 hr. about 90% of the glucose disappearing had been converted into HDP. When HMP was used as substrate, there was no disappearance in the absence of ATP, indicating that no side reactions, such as conversion into 6-phosphogluconic acid, were taking place; in the presence of ATP, HMP was converted almost quantitatively into HDP.

Crane & Sols (1954) have shown that 2-deoxyglucose is phosphorylated by 'particulate' ox-brain hexokinase to give 2-deoxyglucose 6-phosphate. This compound is inactive as a substrate for phosphohexose isomerase or 6-PFK; furthermore, unlike glucose 6-phosphate, it does not inhibit rat-

Table 1. *Utilization of glucose and 2-deoxyglucose by rat-brain extract and yeast hexokinase in the presence of different buffers*

Composition of reaction medium (final concn. in brackets): glucose or 2-deoxyglucose (0·004 M), ATP (0·005 M), $MgCl_2$ (0·0067 M), KF (0·024 M) and buffer as indicated. Rat-brain extract (from a centrifuged 1:8 water homogenate) or yeast hexokinase (stock preparation diluted 1:200), 0·05 ml.; total vol., 0·5 ml.; 20 min. incubation at 30°.

		Rat-brain extract		Yeast hexokinase	
Buffer	Final concn. (M)	Glucose used (μmoles)	2-Deoxyglucose used (μmoles)	Glucose used (μmoles)	2-Deoxyglucose used (μmoles)
Tris	0·025	0·25	1·01	0·71	1·08
$NaHCO_3$*	0·020	0·36	1·07	0·71	—
Potassium phosphate	0·025	0·81	1·09	0·75	0·94

* Equilibrated with N_2+CO_2 (95:5).

Table 2. *Effects of erythrocyte lysate, rat-muscle extract and rabbit-muscle fraction B on the utilization of glucose and 2-deoxyglucose by rat-brain extracts*

Enzyme: rat-brain extract (from a centrifuged 1:8 water homogenate; 0·05 ml.) Activators: human-erythrocyte lysate (washed human erythrocytes, lysed with enough water to restore vol. to that of blood taken; 0·15 ml.); rat-muscle extract (from a centrifuged 1:3 homogenate in 0·03 M-$NaHCO_3$; 0·05 ml.); rabbit-muscle fraction *B* (stock solution diluted 1:5 and dialysed for 1 hr. against 0·25 M tris or potassium phosphate buffer, pH 7·6, at 0°; 0·05 ml.). Enzyme and/or activator incubated for 20 min. at 30° with glucose or 2-deoxyglucose, ATP, $MgCl_2$, KF and buffer, pH 7·6, at concn. shown in Table 1; total vol. 0·5 ml. In all cases, data for enzyme + activator have been corrected for the slight substrate disappearance due to activator alone.

			Glucose utilization			2-Deoxyglucose utilization		
Expt.	Buffer	Activator	No activator (μmoles)	Activator present (μmoles)	Activation (%)	No activator (μmoles)	Activator present (μmoles)	Activation (%)
1	Tris	Human-erythrocyte lysate	0·22	0·54	145	—	—	—
2	Tris	Rat-muscle extract	0·19	0·74	290	0·78	1·20	54
3	Tris	Rabbit-muscle fraction *B*	0·25	0·68	172	0·93	1·24	33
4	Phosphate	Rat-muscle extract	0·43	0·68	58	0·86	1·02	19
5	Phosphate	Rabbit-muscle fraction *B*	0·53	0·74	40	0·99	1·12	13

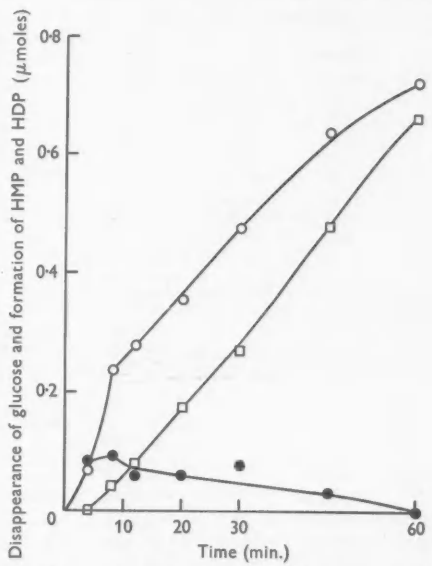

Fig. 1. Disappearance of glucose and formation of HMP and HDP in rat-brain extract. Composition of reaction medium (final concn. in brackets): glucose (0·004 M), ATP (0·005 M), $MgCl_2$ (0·0067 M), tris (0·025 M) and KF (0·024 M). Rat-brain extract (from a centrifuged 1:8 water homogenate), 0·05 ml.; total vol., 0·5 ml.; temp., 30°. ○, Glucose disappearance; ●, HMP formation; □, HDP formation.

brain hexokinase activity. As shown in Table 1, the enzymic phosphorylation of 2-deoxyglucose takes place much more rapidly than that of glucose when the two substrates are compared by using the same rat-brain extract; the difference is most marked when the reaction mixtures are buffered with tris. The rate of phosphorylation of 2-deoxyglucose was not markedly affected by the nature of the buffer.

Yeast hexokinase. With glucose as substrate, the amount disappearing was quantitatively accounted for as HMP; the enzyme preparation was free from 6-PFK activity. The nature of the buffer had no appreciable effect on the rate of enzymic phosphorylation of either glucose or 2-deoxyglucose; the latter was the more active substrate (Table 1).

Effects of activators on the utilization of glucose and 2-deoxyglucose

Rat-brain extract. Weil-Malherbe & Bone (1951*b*, *c*) showed that the rate of glucose utilization by a rat-brain extract could be greatly increased by the addition of a human-erythrocyte lysate or a rabbit-muscle extract, although neither activator had any appreciable hexokinase activity. We have confirmed these observations and find that the rate of utilization of 2-deoxyglucose is also slightly increased in the presence of activators, although the effect produced is only about 20–30% of that obtained with glucose as substrate (Table 2). Rabbit-muscle fraction *B* also showed similar activating properties. Table 2 shows further that the percentage activation of glucose utilization was always much higher in a tris-buffered medium than in the presence of phosphate; the difference is attributable to the increased rate of glucose disappearance in phosphate-buffered media in the absence of activator (Table 1). It may be concluded from these results that the activators exert their effects to some small extent by their direct action on hexokinase, as shown by the data for 2-deoxyglucose; however, with glucose as substrate the much greater activation observed strongly suggests that they possess a quantitatively more important action.

In the enzymic phosphorylation of glucose, the amounts of HMP and HDP formed have been determined in the presence and absence of activators. In these experiments the reaction medium contained tris, since this buffer favoured large activating effects. The data of Table 3 show clearly that the extra glucose disappearing in the presence of activator is largely accounted for by an increased formation of HDP. Of even greater significance, however, is the observation that the three activators studied all bring about a substantial fall in the level of HMP in the reaction medium. Clearly, this action could cause an indirect stimulation of glucose utilization, since glucose 6-phosphate is an inhibitor of rat-brain hexokinase (Weil-Malherbe & Bone, 1951*a*; Crane & Sols, 1953). If the phosphohexose isomerase or 6-PFK activities of the unsupplemented rat-brain extract limits the rate of removal of glucose 6-phosphate, then the presence of either or both of these enzymes in the activator could produce the observed result. It seems unlikely that activation is due to phosphohexose isomerase, however, for Weil-Malherbe & Bone (1951*b*, *c*) have already shown that both erythrocyte lysates and rabbit-muscle extracts stimulate the utilization of fructose as well as of glucose by rat-brain extract, and we have obtained the same result by using rat-muscle extract as activator. These observations suggest that the activation takes place at a stage beyond phosphohexose isomerase, which would implicate 6-PFK. This possibility is supported by the fact that partially purified 6-PFK (rabbit-muscle fraction *B*) can also cause activation, as shown in Tables 2 and 3.

Table 3. *Effects of activators on glucose utilization and HMP and HDP formation in rat-brain extracts*

Experimental conditions as in Table 2. Reaction mixtures were buffered with 0·025 M tris and analysed for glucose, HMP and HDP. In all cases the data for enzyme + activator have been corrected by subtraction of the slight effects due to activator alone.

Expt.	Activator		Changes observed (μmoles) Glucose	HMP	HDP	Activation (%)
1	Human-erythrocyte lysate	Enzyme (*a*)	−0·22	+0·08	+0·18	—
		Enzyme + activator (*b*)	−0·54	+0·01	+0·46	—
		(*b*) − (*a*)	−0·32	−0·07	+0·28	145
2	Rat-muscle extract	Enzyme (*a*)	−0·42	+0·12	+0·19	—
		Enzyme + activator (*b*)	−0·90	+0·06	+0·64	—
		(*b*) − (*a*)	−0·48	−0·06	+0·45	114
3	Rabbit-muscle fraction *B*	Enzyme (*a*)	−0·22	+0·08	+0·18	—
		Enzyme + activator (*b*)	−1·06	+0·01	+0·72	—
		(*b*) − (*a*)	−0·84	−0·07	+0·54	282

Table 4. *Effect of heated and unheated rat-muscle extract and rabbit-muscle fraction B on the utilization of glucose and 2-deoxyglucose*

In Expts. 1–4, rat-brain extract was used; in Expt. 5, the enzyme was yeast hexokinase. Rat-muscle extract or rabbit-muscle fraction *B* was heated for 5 min. at 80°, and the coagulum dispersed by homogenization. Other conditions as in Table 2. The percentage activation is given in brackets.

Expt.	Activator	Buffer	Glucose utilization (μmoles) No activator	Unheated activator	Heated activator	2-Deoxyglucose utilization (μmoles) No activator	Unheated activator	Heated activator
1	Rat-muscle extract	Tris	0·14	1·01 (620)	0·26 (86)	0·67	1·25 (87)	0·81 (21)
2	Rat-muscle extract	Phosphate	0·47	0·76 (62)	0·57 (21)	0·95	1·28 (35)	1·07 (13)
3	Rabbit-muscle fraction *B*	Tris	0·25	0·68 (172)	0·29 (16)	0·93	1·24 (33)	0·94 (1)
4	Rabbit-muscle fraction *B*	Phosphate	0·57	0·91 (60)	0·62 (9)	1·04	1·48 (42)	1·19 (14)
5	Rat-muscle extract	Tris	0·79	1·14 (44)	0·80 (1)	0·95	1·21 (27)	1·00 (5)

Yeast hexokinase. Weil-Malherbe & Bone (1951*b*, *c*) found yeast hexokinase, with glucose as substrate, to be 32% activated by both human-erythrocyte lysates and rabbit-muscle extracts. Since this enzyme is not inhibited by HMP (Berger, Slein, Colowick & Cori, 1946), the effect clearly cannot be related to the presence of 6-PFK in the activators, and is probably of a similar nature to the slight activation of 2-deoxyglucose utilization

found with rat-brain extracts (Table 2), which was regarded as a direct effect on the hexokinase enzyme. We have confirmed the findings of Weil-Malherbe & Bone, using rat-muscle extract as activator, with both glucose and 2-deoxyglucose as substrate (see Table 4, Expt. 5).

Effect of heat treatment on the activators

Weil-Malherbe & Bone (1951*b*, *c*) showed that heating activator solutions for 5 min. at 80° destroyed their 6-PFK activity and diminished but did not completely eliminate their effects on glucose utilization by rat-brain extracts. This observation was taken to indicate that activation was not due to 6-PFK activity. We have repeated these experiments, but find that the heat treatment causes a greater fall in activation than that reported by Weil-Malherbe & Bone. Table 4 shows the results obtained with both glucose and 2-deoxyglucose as substrates. With glucose, the effect of the activator was much reduced by heat treatment, being especially marked when the system was buffered with tris. With 2-deoxyglucose, the heated activators had practically no effect on substrate utilization.

With yeast hexokinase, heated rat-muscle extract had practically no activating effect on the utilization of glucose or 2-deoxyglucose (Table 4).

Activation by heat-denatured serum albumin and EDTA

By the use of rat-brain extract with glucose as substrate, it has been found that activating effects are produced by serum albumin and EDTA (Table 5). It will be observed that the percentage activations caused by EDTA and heat-denatured albumin are of the same order of magnitude as those caused by heat-treated rat-muscle extract. By contrast, it was found that when 2-deoxyglucose was the substrate, EDTA and heat-denatured albumin had little or no activating effect.

6-PFK activities of rat-brain extract and activators

6-PFK activities were measured by incubating rat-brain extract or activator with 0·014 M HMP and 0·01 M ATP, as described in the Methods section, and determining loss of HMP and formation of HDP. This concentration of substrate is more than sufficient to saturate the enzyme (Weil-Malherbe & Bone, 1951*a*), but raising the ATP concentration above 0·01 M did not increase the rate of utilization of HMP. These conditions of assay were not necessarily optimal, but it was clearly desirable to keep them as similar as possible to those used in the activation experiments. Disappearance of HMP was in all cases quantitatively accounted for as HDP, and no dephosphorylation was observed. Relative 6-PFK activities are given in Table 6. Rat-muscle extract and the 1:5-diluted rabbit-muscle fraction *B* caused a utilization of 3–3·5 μmoles HMP/0·05 ml./30 min. Human-erythrocyte lysate and rat-brain extract possessed lower activity. It is noteworthy that the activation of rat-brain hexokinase by erythrocyte lysate is usually rather less than that produced by either rabbit-muscle fraction *B* or rat-muscle extract, which is consistent with the suggestion that the main activator is 6-PFK.

In an experiment with rat-brain extract, activated by either erythrocyte lysate, rat-muscle extract of low 6-PFK activity (caused by filtration

Table 5. *Effects of heat-denatured serum albumin, EDTA and acid-treated activators on the utilization of glucose by rat-brain extract*

Serum albumin solution, denatured by heating for 5 min. at 80°; final concn. 0·5%. EDTA, final concn., 0·001 M. Rat-muscle extract or rabbit-muscle fraction *B* was acidified to pH 6 and left at room temp. for 1 hr., and the pH was then readjusted to 7·6. In Expts. 3 and 4, the figures in brackets denote the 6-PFK activities of the muscle extracts (μmoles HMP utilized/20 min.).

			Glucose utilization (μmoles)		
Expt.	Buffer	Activator	No addition	Activator present	Activation (%)
1	Phosphate	Rat-muscle extract	0·47	0·91	94
		Heated rat-muscle extract	0·47	0·57	23
		Albumin	0·47	0·56	19
		EDTA	0·47	0·67	43
2	Tris	Rat-muscle extract	0·27	0·94	248
		Heated rat-muscle extract	0·27	0·49	81
		Albumin	0·27	0·41	52
		EDTA	0·27	0·43	59
3	Tris	Rat-muscle extract	0·32	1·00 (2·53)	212
		Acid-treated rat-muscle extract	0·32	0·68 (1·17)	115
4	Phosphate	Rabbit-muscle fraction *B*	0·63	1·69 (2·89)	170
		Acid-treated rabbit-muscle fraction *B*	0·63	0·77 (0·05)	22

through a thick pad of kieselguhr), fraction *B* of high 6-PFK activity, and various combinations of these, the degree of activation roughly paralleled the total 6-PFK activity of the added activators (Table 7).

Effects of nature of buffer, EDTA, heated and acidified activators and serum albumin on the 6-PFK activity of rat-brain extracts

The heated activators, heat-denatured serum albumin and EDTA all increase the rate of glucose utilization by rat-brain extracts (Table 5). These effects could be exerted on the hexokinase and/or 6-PFK activities of the brain extract. Table 8 shows that all the substances mentioned above increase the measured 6-PFK activity of rat-brain extracts. The 6-PFK activity of rat-brain extracts is also greater in the presence of phosphate buffer than in tris buffer. Since 6-PFK activity is sensitive to mild acid treatment at room temperature (Taylor, 1947; Utter, 1947), the effect of acidification on the activators was of particular interest. As will be seen from Expts. 3 and 4 of Table 5, the diminution of the activating power of rat-muscle extract and rabbit-muscle fraction *B* paralleled the reduction in 6-PFK activity.

Table 6. *6-PFK activities of rat-brain extract and activators*

Rat-brain extract or activator was incubated with 0·014 M HMP, 0·01 M ATP, 0·0067 M-$MgCl_2$, 0·024 M-KF and 0·025 M tris buffer, pH 7·6, for 20 min. at 30°; total vol. 0·5 ml. Reaction mixtures deproteinized with TCA and filtrates analysed for HMP and HDP.

Source of 6-PFK	Vol. used (ml.)	No. of measurements	Average HMP utilized (μmoles)	Average HDP formed (μmoles)
Rabbit-muscle fraction *B**	0·05	8	3·42	3·41
Rat-muscle extract	0·05	3	3·28	3·27
Human-erythrocyte lysate	0·15	5	1·40	1·33
Rat-brain extract	0·05	7	1·14	1·08

* A 1:5 dilution of stock preparation, dialysed against 0·25 M tris buffer, pH 7·6, for 1 hr. at 0°.

Table 7. *Glucose utilization by rat-brain extract in the presence of activators of different 6-PFK activities*

Rat-brain extract, erythrocyte lysate and rabbit-muscle fraction *B*, as in Table 2; rat-muscle extract was filtered through a thick pad of kieselguhr in order to diminish its 6-PFK activity. Tris buffer used; other conditions as in Table 2. Glucose-utilization values are corrected for the slight hexokinase activities of the activators.

Activator	6-PFK activity of activator (μmoles HMP utilized/20 min.)	Glucose utilization (μmole/20 min.)	Activation (%)
None	—*	0·31	—
Rat-muscle extract (*a*)	0·63	0·41	35
Erythrocyte lysate (*b*)	1·63	0·49	60
(*a*) + (*b*)	2·26	0·62	103
Rabbit-muscle fraction *B* (*c*)	2·46	0·76	149
(*a*) + (*c*)	3·09	0·81	164
(*a*) + (*b*) + (*c*)	4·72	0·85	178

* 6-PFK activity of rat-brain extract alone, 1·90 μmoles HMP utilized/20 min.

Table 8. *Effects of buffer, heated rat-muscle extract, serum albumin and EDTA on the 6-PFK activity of rat-brain extracts*

Experimental conditions as in Table 6. 20 min. incubation at 30°.

Expt.	Buffer	Activator	HMP utilized (μmoles)	HDP formed (μmoles)
1	Tris	None	1·95	1·44
	Phosphate	None	2·56	2·68
2*	Phosphate	None	0·87	0·93
		Heat-denatured albumin	1·26	1·30
		Heat-treated rat-muscle extract	1·37	1·48
		EDTA	1·64	1·78

* A diluted rat-brain extract was used in this experiment.

Stern (1954) has stated that the rate of utilization of glucose by a rat-brain extract, both in the presence and absence of activator, is lowered by the addition of 0·003 M-$CaCl_2$. We have found that under these conditions the 6-PFK activity of the rat-brain extract is diminished, and this could partly explain the effects reported. In addition, however, Ca^{2+} ions are known to form a complex with ATP (DiStefano & Neuman, 1953), so that the reduced activity is probably also partly attributable to this cause.

Inhibition of 2-deoxyglucose utilization in acid-treated rat-brain extract by HMP and stimulation by rat-muscle extract

If activators increase the rate of glucose utilization in rat-brain extracts largely by removal of inhibitory HMP by virtue of their 6-PFK activity, then it should be possible to demonstrate a similar effect with 2-deoxyglucose as substrate in the presence of added HMP. Under these conditions, there would be no formation of HMP during the experiment, in contrast with what would occur with glucose as substrate. In order to prevent the utilization of the added HMP during the course of the experiment, it was necessary to use an acid-treated rat-brain extract of negligible 6-PFK activity. This was obtained by pre-incubation of the rat-brain extract at pH 5·5 and 37° for 15 min. (Racker & Krimsky, 1948), followed by readjustment of the pH to 7·6; in the experiment shown in Table 9, 0·12 μmole of HMP was added initially and 0·11 μmole was still present at the end of 20 min. incubation with the pre-treated rat-brain extract, showing that the 6-PFK activity had been virtually destroyed by the acid treatment. It will be seen that 0·12 μmole HMP, which is of the same order as the amount normally present in rat-brain extracts phosphorylating glucose (Fig. 1 and Table 3), inhibited 2-deoxyglucose utilization by about 47 %. When rat-muscle extract was added, the inhibition almost completely disappeared and the apparent activation was 173 %, compared with 52 % for the uninhibited system.

Table 9. *Effects of HMP and rat-muscle extract, singly and together, on the rate of 2-deoxyglucose utilization by a rat-brain extract free from 6-PFK activity*

Aqueous rat-brain extract, adjusted to pH 5·5 with N-HCl (glass electrode), incubated 15 min. at 37° and pH readjusted to 7·6 with 3·5N-KOH. Other experimental conditions as in Table 2. HMP, 0·12 μmole where indicated.

Additions	2-Deoxyglucose utilized (μmoles/ 20 min.)	Activation (%)
None	0·83	—
Rat-muscle extract	1·26	52
HMP	0·44	—
Rat-muscle extract + HMP	1·20	173

DISCUSSION

The observations first made by Weil-Malherbe & Bone (1951*b*, *c*) that lysates of human erythrocyte and rabbit-muscle extracts were able to increase the rate of glucose utilization by a suitably supplemented rat-brain extract seem now to be satisfactorily explained in terms of the enzymic pathway: glucose → glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate.

The elucidation of the mechanisms involved has been due mainly to the use of 2-deoxyglucose as an alternative substrate for rat-brain hexokinase, since in this way the hexokinase reaction becomes isolated from subsequent stages of the glucolytic pathway. The employment of the spectrophotometric method of Slater (1953) for the specific determination of hexose mono- and di-phosphates and the observations made in the presence of different buffers have also materially helped in investigating this problem.

It seems quite clear that the activators do have a slight effect on the true hexokinase activity of rat-brain extracts. This is shown by the fact that an activation was obtained with 2-deoxyglucose as substrate (Table 2). Activations of the same order of magnitude (20–50 %) were also observed with yeast hexokinase, both glucose and 2-deoxyglucose being used as substrates (Table 4).

These activations are much smaller than those observed by Weil-Malherbe & Bone and by ourselves with rat-brain extract, using glucose as substrate; these usually amounted to about 150–300 % (Table 2), but were sometimes considerably higher. Such high values are traceable to the potent 6-phosphofructokinase contained in the activators; this enzyme acts by causing the rapid removal of fructose 6-phosphate, which consequently reduces the concentration of glucose 6-phosphate, a potent inhibitor of brain hexokinase. The main evidence in support of this explanation is as follows: (1) the activators lower the concentration of hexose monophosphate; (2) they convert most of the extra glucose utilized into fructose 1:6-diphosphate; (3) they have high 6-PFK activities; (4) their effect on the rate of glucose utilization is roughly proportional to their 6-PFK activity; (5) they remove the inhibition of 2-deoxyglucose utilization caused by added HMP; and (6) any treatment which lowers their 6-PFK activity also lowers their activating effects.

Weil-Malherbe & Bone (1951*b*, *c*) had earlier concluded that 6-PFK was not responsible for the observed effects, since heat treatment of the activators destroyed the 6-PFK activity without eliminating completely their effects on glucose utilization. This is now explained by the fact that the heat-denatured activators stimulate the 6-PFK activity of the rat-brain extracts (Table 8) and so produce a qualitatively similar effect, though of lesser magnitude; we have observed these effects also with denatured serum albumin and with EDTA. Thus there appear to be three mechanisms of activation: (1) direct stimulation of hexokinase activity; (2) addition of 6-PFK contained in the activator solutions; (3) stimulation of the 6-PFK activity of the rat-brain extract. In general, of the total stimulation of glucose utilization due to the addition of activator, 20% was caused by (1), 60% by (2) and 20% by (3).

It will be noted (Table 4) that the rate of utilization of glucose by a rat-brain extract supplemented by addition of an activator is always less than the rate with 2-deoxyglucose as substrate under otherwise identical experimental conditions. Crane & Sols (1954), however, have stated that the two substrates have equal maximal rates of utilization with particulate ox-brain hexokinase, when the substrate concentrations are sufficient to saturate the enzyme, as in the present work. The explanation of this discrepancy may be traceable to the inhibition of rat-brain hexokinase by ADP (Weil-Malherbe & Bone, 1951*a*; Sols & Crane, 1954), the concentration of which would be greater, with glucose as the substrate than with 2-deoxyglucose. On the other hand, it is possible that the 6-PFK activity of the activators was insufficient to ensure maximal glucose utilization.

The stimulating effect of phosphate ions on the utilization of glucose by rat-brain extract (Table 1) seems undoubtedly due to the increased 6-PFK activity in this buffer (Table 8); such an explanation also accounts for the fact that the effects of the activators, both heated and unheated, were always uniformly less when this buffer was employed in place of tris (Table 4). Phosphate has no appreciable effect on hexokinase itself, as shown with rat brain extract with 2-deoxyglucose, and with yeast hexokinase with either glucose or 2-deoxyglucose.

SUMMARY

1. The 'activation' of glucose utilization in rat-brain extracts by erythrocyte lysates and rat-muscle extracts has been studied.

2. A slight direct activation of rat-brain hexokinase was observed when 2-deoxyglucose was used as substrate; similar effects were found with the yeast enzyme, with both glucose and 2-deoxyglucose as substrates.

3. Quantitatively the most important effect is due to the increased 6-phosphofructokinase activity of the enzyme system, brought about by addition of activator. This acts by removing inhibitory HMP from the reaction medium.

4. A third effect is exhibited by the stimulation of the 6-phosphofructokinase activity of the rat-brain extract by heat-denatured activators, none of which possessed any detectable 6-PFK activity. Similar effects were produced by heat-denatured albumin, EDTA and phosphate ions.

Our thanks are due to Professor M. Stacey, F.R.S., for a generous gift of 2-deoxyglucose, to Dr E. C. Slater for advice on the preparation of the rabbit-muscle enzymes used in the spectrophotometric determination of hexose mono- and di-phosphates, to Miss Dorothy M. Fraser for blood samples and to Miss Iris Creighton for technical assistance. Part of this work was carried out during the tenure by A.R.T. of a grant from the D.S.I.R., to whom our gratitude is also expressed.

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The Relation between the Synthesis of Deoxyribonucleic Acid and the Synthesis of Protein in the Multiplication of Bacteriophage T2

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Cohen & Fowler (1947) and Cohen (1948) showed that 5-methyltryptophan, which inhibits the biosynthesis of tryptophan (Beerstecher, 1954), can prevent the development of bacteriophage and the net synthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* infected with bacteriophage T2, indicating that the synthesis of DNA is, in this system, dependent on the synthesis of protein. This paper is concerned with a further study of this relationship between the two synthetic processes. In some of the experiments, 5-methyltryptophan has been used to inhibit the synthesis of tryptophan and hence of protein. In most experiments, however, bacterial mutants which require either histidine, leucine, tryptophan, tyrosine or purines for growth have been used as host cells for the bacteriophage. The primary effect of a deficiency in any of the four amino acids is expected to be the inability to synthesize proteins.

Bacteriophage T6r$^+$ was used in the earlier experiments, but in the multiply infected cultures necessary for the metabolic studies considerable difficulty was experienced owing to a variable and unpredictable premature lysis which occurred without liberation of phage (cf. Hershey, Dixon & Chase, 1953). With the closely related bacteriophage T2r this phenomenon is much less frequent, and this bacteriophage was therefore used for the majority of the experiments.

MATERIALS AND METHODS

Bacteria. Esch. coli B and its nutritional mutants were used. The tyrosine-requiring strain was isolated by Dr J. Ceithaml of the University of Chicago from ultraviolet-irradiated cultures of *Esch. coli* B/1. The tryptophan-requiring strain (B/1, *t*) was obtained from Dr A. Novick of the University of Chicago and is a mutant of *Esch. coli* B selected by its resistance to bacteriophage T1. B-33 (leucine$^-$), B-62 (histidine$^-$), B-38 (adenine$^-$) and B-96 were obtained from Dr J. S. Gots of the University of Pennsylvania. Strain B-96 is unable to synthesize purines and accumulates 4-amino-5-imidazolecarboxamide and its derivatives in the growth medium (Gots, 1950, 1953); adenine, guanine, hypoxanthine or xanthine can satisfy the purine requirement of this strain.

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Bacteriophage. The T2r and T6r$^+$ strains did not require tryptophan for adsorption to the host cell. The stocks were obtained by confluent lysis of *Esch. coli* B (cf. Adams, 1950) on the surface of agar medium in 9 cm. diameter Petri dishes. Two layers of agar were used; the lower layer (20 ml.) contained 0·8% Bacto-tryptone (Difco), 0·5% NaCl and 2% agar. The upper layer (3 ml.), which was seeded with about 5×10^7 bacteria and about 10^4 phage, contained the same amounts of tryptone and NaCl but only 0·8% agar. The bacteriophage was purified by two cycles of differential centrifuging (Kozloff & Putnam, 1949).

5-Methyltryptophan. This was kindly supplied by Dr S. S. Cohen.

Infective centres. Infective centres (i.e. plaque-forming particles) were determined after suitable dilution in buffered saline, a double-layer technique (cf. Adams, 1950) being used. The agar layers were similar to those used in the preparation of the bacteriophage and the same amount of bacteria (5×10^7 cells) was added to the upper layer. The plaque count does not differentiate between free phage particles and infected cells which are capable of liberating phage.

Growth media. 'Lactate' medium was the 'F' medium of Cohen & Anderson (1946). It contained sodium lactate and NH_4Cl as sole sources of carbon and nitrogen respectively. 'Mannitol' medium was similar, except that the sodium lactate was replaced by 1% mannitol plus 0·1 M-NaCl. Individual L-amino acids were added as required for the appropriate mutants at the following concentrations: tyrosine, 100 mg./l.; histidine–HCl, 10 mg./l.; leucine, 50 mg./l. The purine-requiring mutants were grown with either adenine (7 mg./l.) or adenosine (15 mg./l.) as a supplement.

The mannitol medium was very suitable for experiments involving multiply infected cells (about 5 phage per bacterial cell) since there was little tendency for premature lysis. The medium was not, however, suitable for experiments with low multiplicities of infection (<0·05 phage per cell) as the singly infected cells gave very variable yields and latent periods. In the lactate medium, although premature lysis tended to occur on multiple infection, the yields and latent periods were more reproducible. In general, therefore, the lactate medium was used for experiments at low multiplicities of infection, and the mannitol medium for experiments involving multiply infected cells.

However, the mannitol medium, when supplemented with acid-hydrolysed casein (0·5% Difco casamino acids) and adenosine (15 mg./l.), gave reproducible phage yields and latent periods, and premature lysis did not follow multiple infection. Since this fortified medium was deficient in tryptophan it was used for all the experiments

with the tryptophan-requiring strain and is designated as 'fortified mannitol medium'. To compensate for the NaCl present in the casein hydrolysate, the amount of NaCl added in making up this medium was only 0·084 M, instead of 0·10 M in the unfortified mannitol medium.

Under conditions of multiple infection with T2r in the lactate or unfortified mannitol media, lysis was often delayed for 1 or 2 hr., although mature intracellular phage appeared at about 18 min. after infection. In the fortified mannitol medium there were visible signs of lysis (intense frothing followed by progressive clearing of the culture) at about 35 min. after infection provided that there was no interference with the phage multiplication by nutritional deficiency.

Buffered saline. This contained 5·85 g. NaCl, 1·5 g. KH_2PO_4, 3·4 g. Na_2HPO_4 and 10 mg. $MgSO_4$ per l.

Growth conditions. For each experiment, bacteria were freshly grown at 37° with good aeration to about 7×10^8 cells/ml. The medium used for the development of the infected cells was identical with that used for the growth of the host bacteria, except for the absence of the growth factor or the presence of the analogue. In all experiments where the behaviour in the absence of a growth factor was compared with that in its presence, a culture was washed and divided into two portions, to one of which the growth factor was added. Cultures were not aerated if the bacterial concentration was less than 10^8 cells/ml.; it was sufficient that the medium had previously been saturated with air.

Washing of cells. This was performed at 4° and required 30–45 min. The culture was centrifuged at 4000 rev./min. for 5 min. in the Servall-type SS-1 centrifuge, the pellet suspended in the original volume of buffered saline, centrifuged and then resuspended. The resuspending fluid was buffered saline if the cells were to be infected with a low ratio (<0·1) of phage particles to bacterial cells. In order to minimize premature lysis when multiply infected cells were required the resuspending medium was similar to the original growth medium except that it lacked the appropriate amino acid or purine.

After decanting the supernatant fluids from the pellets, excess of fluid was removed from the internal walls of the centrifuge tube by means of absorbent tissue.

One-step growth experiments. Washed bacteria (about 7×10^8 viable cells/ml.) were infected in buffered saline with 10^7 bacteriophage per ml. and 5 min. at 37° allowed for adsorption. Measured portions were then diluted 10^3-fold into two tubes of medium, one containing and one lacking the required growth factor. In studying the delayed addition of the growth factor tenfold dilutions were subsequently made from both tubes into fresh medium which, when necessary, contained the growth factor. Bacteriophage liberation was followed by plaque count.

Under these conditions the bacteriophage are liberated at a very low concentration of bacterial cells, so that the rate of adsorption on the uninfected cells present is very slow. A single cycle of bacteriophage growth is thus studied.

Ultraviolet irradiation experiments. Cells were infected as in the one-step growth experiments and then cooled in an ice bath. Samples were diluted 50-fold into growth media from which, at intervals, 0·1 ml. samples were diluted 20-fold into buffered saline and immediately exposed to ultraviolet light in Petri dishes of 9 cm. diameter. During exposure the dishes were gently shaken on the horizontal platform of a rotary shaker; the platform was 33 cm. below a 15 w germicidal lamp and the exposure time was usually 7 sec. The infective centres surviving the irradiation were determined by plaque count.

Chemical analyses

Nucleic acid. A 10 ml. sample of the culture containing about 5×10^9 cells was mixed with 0·22 ml. 70% (w/w) $HClO_4$. After about 2 hr. at 4°, it was centrifuged in a tapered centrifuge tube and the precipitate resuspended in 3 ml. water. N-$HClO_4$ (0·75 ml.) was then added and the sample was recentrifuged after about 30 min. at 4°. The pellet was twice extracted with 4 ml. 0·5 N-$HClO_4$ for 10 min. at 70° and the extracts, which contained the nucleic acids, were combined.

DNA was estimated by mixing 2 ml. of the extract with 0·1 ml. 0·07 M acetaldehyde and 4 ml. diphenylamine reagent (7·5 g. steam-distilled diphenylamine dissolved in 500 ml. acetic acid plus 7·5 ml. conc. H_2SO_4). After 16–25 hr. at 25–30° the optical density was read in a Beckman spectrophotometer at 600 mμ. and compared with that given by calf-thymus DNA of known phosphorus content. The standard DNA was heated for 15 min. at 70° in 0·5 N-$HClO_4$ before mixing with the reagent. The intensity of colour is very nearly proportional to the amount of DNA present; 0·2 μmole DNA phosphorus or 0·1 μmole 2-deoxy-L-ribose results in an optical density of about 0·35 at 600 mμ. This modified diphenylamine reaction is an improvement over the usual procedure (Dische, 1930) involving heating with the reagent at 100° for 10 min. in that it is more sensitive and specific and is much less susceptible to interference by other compounds. Five mg. of a commercial yeast RNA preparation gave the same reading as 0·011 mg. DNA, and 4 mg. each of several substances, including fructose, sucrose, starch, glycogen, glucosamine, ascorbic acid, rhamnose, ribose, acid-hydrolysed casein and tryptophan, gave less colour than 1·5 μg. DNA. Except for ascorbic acid, which suppresses the colour development, none of these compounds influenced the amount of colour obtained from DNA. The method is at least as sensitive and specific as those of Cohen (1944), Stumpf (1947) and Ceriotti (1952). Further properties of the reaction will be described in a later publication.

The results are expressed as moles DNA-nucleotide by comparison with calf-thumus DNA. Diphenylamine appears to react with the sugar derived from the purine nucleotides of the DNA, but not with that derived from the pyrimidine nucleotides, since the latter are not hydrolysed under the conditions used, whereas purine deoxyribose derivatives are very labile to acid. DNA's of different origins, including calf thymus, *Esch. coli* and T2 bacteriophage, all have a purine/pyrimidine ratio very close to unity (Chargaff, 1955); they are therefore all expected to give the same amount of colour with diphenylamine for the same amount of DNA nucleotide.

The RNA values are obtained by the orcinol method (Ogur & Rosen, 1950), with ribose as a standard and a heating time of 45 min. at 100°. To make them comparable with the DNA values they are arbitrarily expressed as RNA nucleotide on the assumption that 1 mole ribose is equivalent to 2 moles RNA nucleotide.

Purines and pyrimidines. These were determined in the DNA of the cultures by the procedure of Hershey *et al.* (1953). The procedure involves separation of the DNA

fraction after alkaline degradation of the RNA, hydrolysis by 6N-HCl followed by paper chromatography and spectrophotometric analysis of the bases.

RESULTS

Amino acid requirements for bacteriophage development

One-step growth experiments showed that each of the four amino acid-requiring mutants did not support the growth of T2r or of T6r$^+$ in the absence of the required amino acid. In the presence of the amino acid the latent period and burst sizes were similar to those obtained with the wild-type strain.

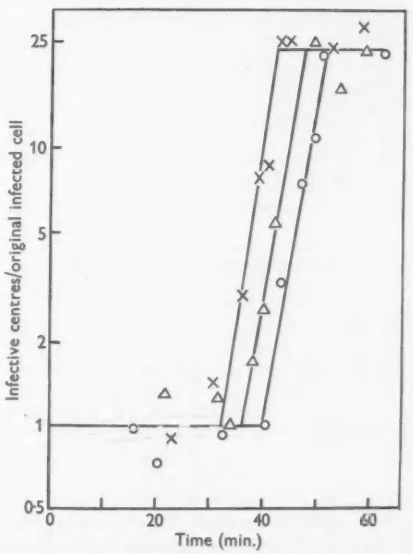

Fig. 1. Phage liberation in one-step growth experiment from tyrosine-requiring *Esch. coli* infected with phage T6r$^+$ in the absence of tyrosine. ×, L-Tyrosine (0·1 g./l.) added at time of diluting into growth medium (0 min.); △, tyrosine added at 5 min.; ○, tyrosine added at 11 min. Lactate medium.

Delayed addition of required amino acids. In experiments with the mutants requiring tyrosine and tryptophan (Figs. 1 and 2), the washed cells were infected with phage in buffered saline and then diluted into growth medium. If the required amino acid was initially absent but added 5 or 10 min. later, the liberation of progeny bacteriophage was delayed by almost the same time as that for which the amino acid had not been present. The results shown in Figs. 1 and 2 suggest that during a period of 10 min. in the absence of the amino acid less than the equivalent of about 2 min. of normal bacteriophage development takes place, despite the presence of all other essential nutrients; development appears to continue normally when the amino acid is subsequently supplied. If a required amino acid is withdrawn after the infected cells have been in a complete medium for several minutes, there is again a suspension of phage development which is resumed on later addition of the amino acid. In the experiment shown in Fig. 3 the tyrosine-requiring mutant was infected and allowed to develop in complete medium for 5 min. before separating the cells and the amino acid by centrifuging. Tyrosine was required for the subsequent liberation of phage. When tyrosine was added after a period of 15 min. in medium lacking

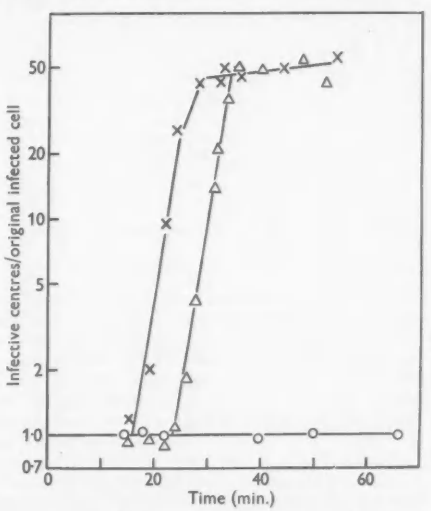

Fig. 2. Phage liberation from tryptophan-requiring *Esch. coli* infected with phage T2r in the absence of tryptophan. ×, L-Tryptophan (50 mg./l.) added at time of diluting into growth medium (0 min.); △, tryptophan added at 10 min.; ○, no tryptophan. Fortified mannitol medium.

tyrosine, the release of phage was, in most of the cells, delayed by about 15 min.

These results are analogous to those of Cohen & Fowler (1947) on the action of 5-methyltryptophan. The inhibition of phage and bacterial growth due to this compound are reversed by tryptophan in accordance with the concept that the analogue inhibits the biosynthesis of tryptophan.

Delayed addition experiments, while very suggestive are, however, ambiguous since the same results could be obtained by partial development in the absence of the amino acid followed by a slower

than normal development when the amino acid was added. For this reason the ultraviolet sensitivity of the infected cells was studied as another indication of their development.

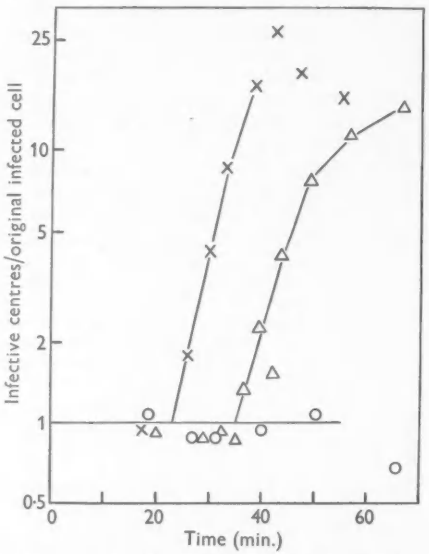

Fig. 3. Phage liberation from tyrosine-requiring *Esch. coli* which had developed in complete medium for 5 min. after infection with phage before withdrawal of the tyrosine. 10^9 cells/ml. in buffered saline at 37° were allowed to adsorb $1{\cdot}5 \times 10^7$ T2r/ml. for 5 min. One volume of lactate medium containing L-tyrosine (8 mg./l.) was added and after 5 min. aeration at 37° the culture was centrifuged at 4° and re-suspended in fresh lactate medium at 37°. ×, L-Tyrosine (0·1 g./l.) added at time of re-suspending (0 min.); △, tyrosine added at 15 min.; ○, no tyrosine. Without centrifuging the infected cells, phage liberation started at 28 min. after infection.

Ultraviolet sensitivity of infected cells

Irradiation of phage-infected cells with ultraviolet light reduces the number of cells which can yield bacteriophage, the radiation sensitivity depending on the state of development at the time of irradiation (Luria & Latarjet, 1947). After the doses of irradiation used in the present experiments, between 12 and 25 % of the infected cells are able to yield phage if irradiated immediately after the infection with phage. During the first 5 min. of development in growth medium the cells start to become much less sensitive. If irradiated with the same doses after 20–25 min. in the growth medium, more than 95 % of the infected cells are able to yield phage. As shown by Luria & Dulbecco (1949) the increase in the resistance to ultraviolet light is greatest in cultures which have been infected with a low ratio (0·01–0·02) of phage to bacteria. The uninfected cells which are present do not interfere in the experiment since the plaque-counting technique scores only free phage or infected cells capable of liberating phage.

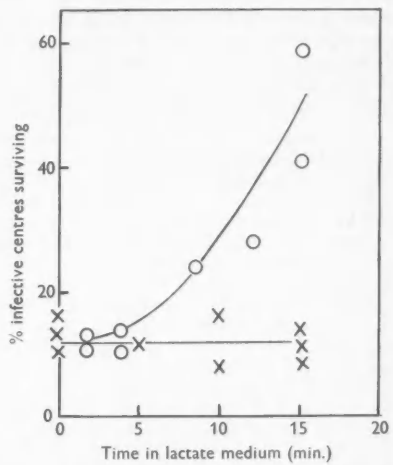

Fig. 4. Effect of ultraviolet light on T6r$^+$-infected cells of tyrosine-requiring *Esch. coli* following different periods of development in lactate medium after infection. ○, Development in the presence of L-tyrosine (0·1 g./l.); ×, no tyrosine. For procedure see text under 'Materials and Methods'.

With each of the four amino acid-requiring mutants used in this work, the appropriate amino acid has been found to be essential for the change in ultraviolet sensitivity of cells infected with either the T2r or the T6r$^+$ bacteriophage (e.g. Fig. 4). This is taken to indicate that an early stage in the development of phage-infected cells requires these four amino acids. The change of sensitivity requires a source of metabolic energy for the infected cells (Benzer, 1952; Benzer & Jacob, 1953).

It should be noted that Luria & Latarjet (1947) consider that the primary effect of ultraviolet irradiation is on the intracellular phage rather than on the host cell. Perhaps the most striking evidence for this view is that bacteria which have been killed by ultraviolet irradiation are still capable of supporting phage growth. Immediately after infection with a single T2 bacteriophage, the ability of the cells to form phage is much more susceptible to the irradiation than is the viability of the uninfected cells.

Adsorption and invasion

Since amino acids are needed early in the infective cycle it must be considered whether the adsorption of the infecting phage and the introduction of viral DNA into the host cell require a supply of amino acids. In most of the above experiments, it is clear that the primary attachment of the phage to the bacterial cell (Puck, 1953) must have occurred before dilution into the growth medium containing amino acids. Otherwise, the rate of attachment in the diluted cultures would have been too slow for any appreciable development to occur.

Hershey & Chase's (1952) experiments on the introduction of the phage DNA into the host cell do not exclude an amino acid requirement for this step, because the bacteria had not been washed free of the nutrient broth in which they had been grown. To investigate this possibility the tyrosine-requiring mutant was grown in mannitol medium supplemented with tyrosine, washed and resuspended at 10^9 cells/ml. in medium lacking tyrosine. Bacteriophage T2r (2×10^8/ml.) were then added. After 4 min. at 37° for adsorption, a sample was diluted with 20 vol. of mannitol medium at 0° and treated in the Waring Blendor for 5 min. No infective centres were destroyed. In another experiment, the conditions of Hershey & Chase (1952) were followed except that the tyrosine mutant was used and washed in buffered saline before infecting with phage. After treatment in the Blendor for 8 min., 90 % of the original number of infective centres were sedimentable at 2000 *g* in 10 min. and 7 % remained in the supernatant. The infective centres therefore consisted of infected bacteria and not of free phage.

The shearing forces produced in the Blendor remove the bulk of the phage protein from infected cells within a few minutes, so that, if the infective portion of the phage had not entered the cell it would have been removed with the phage protein. This would have caused a loss of sedimentable infective centres.

In another experiment, which essentially followed the procedure of Anderson (1953), it was shown that, after infecting the histidine-requiring *Esch. coli*, 80 % of the infective centres became resistant to osmotic shock in 5 min., independent of the presence of histidine. In this experiment, the washed bacteria (7×10^8 cells/ml.) were suspended in a mixture of 4 vol. mannitol medium and 1 vol. glycerol before infecting with T2r (0·04 phage per bacterium) which had previously been incubated in the glycerol-containing medium for 30 min. at 37°. Samples were subjected to osmotic shock by rapid dilution into 100 vol. of water. At least 99 % of the free phage are inactivated by this treatment and the resistance which develops in the presence of the bacteria is presumably an indication of the entry of the DNA of the infecting bacteriophage into the host cell.

Effects of purine deficiency

Delayed addition (Figs. 5, 6) and ultraviolet sensitivity experiments with the purine-requiring mutants (Fig. 7) showed that an external source of purine was not required for the early stages of development. In some experiments, particularly with strain B-96, there was a small yield of phage (average of 2–4 per cell) in the absence of a purine or available derivative.

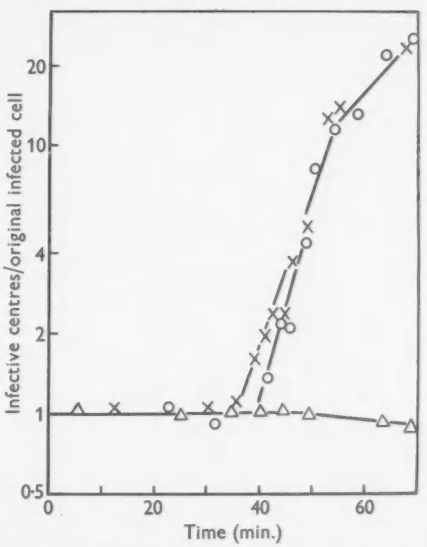

Fig. 5. Phage liberation in one-step growth experiment from purine-requiring *Esch. coli* (B-96) infected with phage T6r$^+$ in the absence of purines. ×, Adenine (7 mg./l.) added at time of diluting into growth medium, (0 min.); ○, adenine added at 11·5 min.; △, no adenine. Lactate medium.

With strain B-96 in mannitol medium, the amounts of RNA and DNA were found to remain constant in the absence of purines whether the bacteria were infected with phage or not. The results with strain B-38 (adenine$^-$) were somewhat variable, but in all cases they indicated an appreciable development in the absence of a source of adenine. However, sometimes, as in Fig. 7, the development appeared to be slower in the absence of adenine, while in other experiments the increase in the resistance to ultraviolet light was similar to that obtained in the presence of adenine.

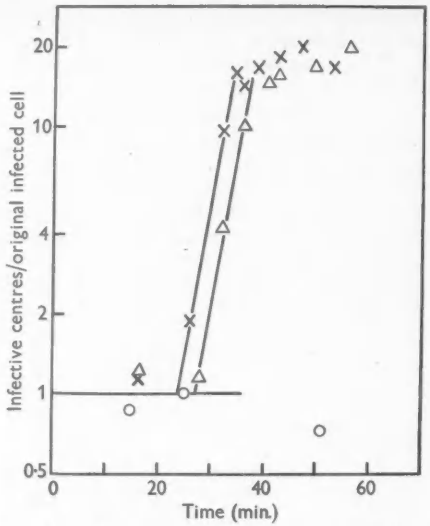

Fig. 6. Phage liberation from adenine-requring *Esch. coli* (B-38) infected with phage T2r in the absence of adenine. ×, Adenine (7 mg./l.) added at time of diluting into growth medium (0 min.); △, adenine added at 10 min.; ○, no adenine. Lactate medium.

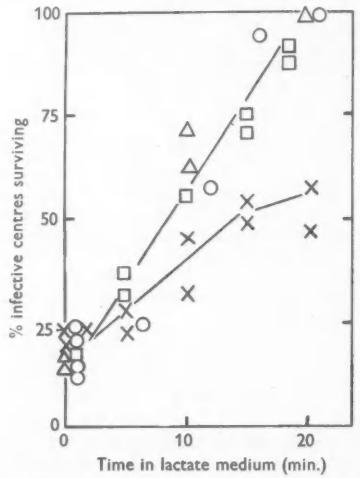

Fig. 7. Effect of ultraviolet light on T2r-infected cells of purine-requiring *Esch. coli* following different periods of development in lactate medium after infection. △, Strain B-96, development in the presence of adenine (7 mg./l.); ○, B-96, no adenine; □, B-38 with adenine; ×, B-38, no adenine. For procedure see text under 'Materials and Methods'.

Metabolism of bacteria infected with bacteriophage

In studying the chemical changes in phage-infected bacteria it is desirable that practically all the cells should be infected with bacteriophage; this is not necessary, and indeed is often not desirable, in experiments such as those described above where the development of infected cells is studied by techniques which are not interfered with by the presence of uninfected cells. The following experiments were therefore performed with multiplicities of infection (ratio of infecting phage particles to bacterial cells) of between 5 and 10 and viable counts were routinely performed before and after adding the phage to check that at least 98 % of the cells were killed by the bacteriophage. Plaque counts were also performed when adsorption was complete and the number of infective centres were usually found to be within 10 % of the original number of viable bacterial cells.

Respiration. The O_2 consumption of the tyrosine-requiring mutant in the mannitol medium was followed for 30 min. after the infection with phage T2r and was found to be independent of the presence of tyrosine. Uninfected cells in the

Table 1. *Tyrosine requirement for synthesis of deoxyribonucleic acid by phage-infected tyrosine-requiring* Esch. coli

Washed cells (5×10^8/ml.) in mannitol medium infected with 5×10^9 T2r/ml. at 0 min.

Time (min.)	DNA (μmoles nucleotide/100 ml.) No tyrosine	DNA (μmoles nucleotide/100 ml.) L-Tyrosine (40 mg./l.)
0	3·68	3·23
5	3·34	3·35
8	—	3·87
12	3·68	4·40
17	—	4·93
22	3·34	6·10
40	3·80	8·71

Table 2. *Tryptophan requirement for synthesis of deoxyribonucleic acid by phage-infected tryptophan-requiring* Esch. coli

Washed cells (4×10^8/ml.) in fortified mannitol medium infected with 3×10^9 T2r/ml. at 0 min.

Time (min.)	DNA (μmoles nucleotide/100 ml.) No tryptophan	DNA (μmoles nucleotide/100 ml.) L-Tryptophan (10 mg./l.)
0	2·47	2·57
5	2·64	2·55
15	2·55	4·51
35	2·53	6·82
45	2·61	8·9
55	2·68	—

absence of tyrosine respired at the same rate as a similar culture immediately after the addition of tyrosine. However, in the presence of tyrosine there was of course an increase in the rate of O_2 consumption in parallel with the cell multiplication. As shown by Cohen & Anderson (1946), cells infected with bacteriophage T2 respire at the same rate as uninfected cells; the respiration of infected cells is constant until the time of lysis.

Nucleic acid synthesis. Cohen (1948) showed that there is no net synthesis of RNA in *Esch. coli* infected with bacteriophage T2 and that the net synthesis of DNA, although suspended for about the first 8 min., soon occurs at a faster rate than the synthesis of DNA in comparable uninfected cultures. As shown in Tables 1 and 2, T2-infected cultures of the mutants requiring tyrosine or tryptophan did not synthesize DNA unless the appropriate amino acid was present. In these and in most similar experiments, the amount of RNA remained constant until the time of lysis. In some cultures, however, the RNA decreased by 20–30% of the initial content, and the decrease in turbidity indicated that premature lysis had occurred.

In contrast to the behaviour of infected cells, uninfected cells continued to synthesize both RNA and DNA in the absence of a required amino acid, but at reduced rates (Table 3). Sands & Roberts (1952) studied the incorporation of ^{32}P into the nucleic acids of a mutant of *Esch. coli* which required both histidine and tryptophan. In qualitative agreement with the results of Table 3, Sands & Roberts found that, in the absence of one or both of the required amino acids, the incorporation of ^{32}P into the RNA and the DNA fractions was reduced but not completely abolished.

In the previous section of this paper, it was shown that tyrosine was necessary for the development of bacteriophage not only in the first few minutes after infection but also at later times. Cohen & Fowler's (1947) experiments with 5-methyltryptophan also indicate that amino acids are essential throughout the period of bacteriophage development, even as late as the time at which mature intracellular phage normally appears. The following experiments show that the synthesis of DNA does not require tyrosine or tryptophan during the later stages of the infective cycle.

The mutant strains of bacteria were infected with T2r in the presence of the required amino acid, which was removed by centrifuging at about 5 min. after the infection. The synthesis of DNA continued at almost the same rate in the absence of the amino acid as when the amino acid was supplied (Table 4). However, as expected from experiments with singly infected cells, the amino acid was essential for the appearance of mature phage. The values for the infective centres shown in Table 4 were obtained both in the usual way after diluting in buffered saline and also after cyanide treatment. This involved dilution in buffered saline containing 0·01 M-NaCN to about 1000 infected cells per ml. and heating at 45° for 5 min. before taking samples for plaque counting. The cyanide treatment does not affect free phage but causes a large proportion of the infected cells to lyse and liberate any mature phage they may contain. An increase in the infective centres due to the cyanide treatment therefore indicates the presence of infected cells containing more than one phage particle per cell. Infected cells which do not contain phage will not give rise to plaques if they are lysed by the cyanide treatment; a fall in the infective centres due to the cyanide treatment therefore indicates that the infected cells contain an average of less than one phage per cell. Cells which do not contain phage are not so readily lysed in the cyanide as those which do contain phage.

Table 3. *Tyrosine and the synthesis of nucleic acids in tyrosine-requiring* Esch. coli

Washed bacteria were suspended in fresh mannitol medium (8×10^8 cells/ml.) at 0 min.

Time (min.)	DNA (μmoles nulceotide/100 ml.)		RNA (μmoles nucleotide/100 ml.)	
	No tyrosine	L-Tyrosine (0·1 g./l.)	No tyrosine	L-Tyrosine (0·1 g./l.)
0	3·6	3·6	10·5	10·3
20	4·1	4·6	11·8	13·2
30	4·6	5·3	13·2	14·0
40	5·0	5·7	14·2	15·8

Results analogous to those in Table 4 were obtained by adding 5-methyltryptophan at various times after infecting the wild-type strain with bacteriophage (Fig. 8). The rate of DNA synthesis with 5-methyltryptophan added 7 min. after the infection was 50–60% of the rate of synthesis observed in the absence of the analogue. In the presence of 5-methyltryptophan, lysis did not occur within the experimental period of 2 hr. and no liberation of mature phage was detected. As

Table 4. *Deoxyribonucleic acid synthesis and bacteriophage formation when amino acids required by host bacteria are withdrawn from phage-infected cells*

Expt. 1. Tyrosine-requiring *Esch. coli* (9×10^8 cells/ml.) washed and infected at 37° with 4×10^9 T2r/ml. in aerated mannitol medium containing 3 mg./l. L-tyrosine. After 5 min. centrifuged at 4° and re-suspended in fresh medium. *A* with L-tyrosine (40 mg./l.); *B* no tyrosine.

Expt. 2. Tryptophan-requiring *Esch. coli* (9×10^8/ml.) washed and infected with 5×10^9 T2r/ml. in aerated fortified mannitol medium containing 1 mg./l. L-tryptophan. After 3·5 min. centrifuged and re-suspended in fresh medium. *A* with L-tryptophan (10 mg./l.); *B* no tryptophan.

Time after resuspending (min.)	DNA (μmoles nucleotide/100 ml.)		Bacteriophage plaque count (infective centres/10^{-11} l.)			
			No CN^-		Treated CN^-	
	A	*B*	*A*	*B*	*A*	*B*
			Experiment 1			
0	2·60	2·56	—	2·94	—	1·8
3	3·15	2·80	—	—	—	—
6	3·23	3·20	—	—	3·5	1·5
10	3·69	3·60	—	—	—	—
12	—	—	—	—	25	1·4
16	4·74	4·31	—	—	—	—
20	—	—	9·2	2·6	—	0·55
25	6·66	5·31	—	—	—	—
35	6·94	5·74	20	2·8	28	0·72
			Experiment 2			
0	2·96	3·13	2·9	2·9	2·2	2·2
3·5	3·45	3·17	—	—	—	—
6·5	4·87	3·91	—	—	—	—
9	—	—	3·3	3·4	2·6	2·2
10·5	5·72	4·17	—	—	—	—
14·5	7·1	4·79	24	2·8	120	0·3
21	—	—	—	2·1	150	0·63
26	8·5	5·16	—	—	—	—
28	—	—	52	3·4	55	—
35	9·3	6·4	40	3·4	50	0·76

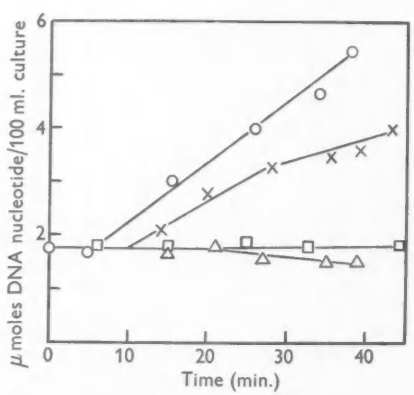

Fig. 8. Effect of 4×10^{-4} M DL-5-methyltryptophan on the synthesis of deoxyribonucleic acid in phage-infected *Esch. coli* B. 4×10^8 cells/ml. in mannitol medium, infected with $1{\cdot}5 \times 10^9$ T2r/ml. at 0 min. ○, No 5-methyltryptophan; 5-methyltryptophan added 3 min. (△) before phage, 1 min. (□) after phage and 7 min. (×) after phage.

previously shown by Cohen (1948), when the inhibitor was added before the infecting phage there was no net synthesis of DNA.

Appearance of 5-hydroxymethylcytosine in the DNA

The DNA of T2 bacteriophage contains 5-hydroxymethylcytosine (Wyatt & Cohen, 1953) and no cytosine, whereas the bacterial DNA, like all other DNA samples which have been examined, contains cytosine but no 5-hydroxymethylcytosine. It was therefore of interest to estimate the 5-hydroxymethylcytosine in the DNA fraction of infected cells under the various conditions (Table 5). It was found that, if tryptophan had been removed from strain B/1,*t*, or if 5-methyltryptophan had been added to the wild-type strain before infection, there was virtually no appearance of 5-hydroxymethylcytosine, and as shown in the above experiments there was no DNA synthesis. When development was allowed to continue normally for some minutes after phage infection before interfering with the availability of tryptophan, the DNA which was synthesized contained 5-hydroxymethylcytosine, and the amount of cytosine

Table 5. *Synthesis of deoxyribonucleic acid containing 5-hydroxymethylcytosine*

Expt. 1. *Esch. coli* B (9×10^8 cells/ml.) in mannitol medium infected with 6×10^8 T2r/ml. at 0 min. 4×10^{-4} M DL-5-Methyltryptophan added as indicated.
Expt. 2. *Esch. coli* B/1, † (6×10^8 cells/ml.) in fortified maannitol medium infected with 5×10^8 T2r/ml. at 0 min. DL-Tryptophan (10 mg./l.) present as indicated.

	Time (min.)	DNA (μmoles nucleotide/100 ml. culture)		Bases in DNA fraction after hydrolysis (μmoles/100 ml. culture)					
		Total	In DNA-fraction	Adenine	Cytosine	Guanine	5-Hydroxymethylcytosine	Thymine	Total
			Experiment 1						
Uninfected bacteria	—	2·38	2·26	0·40	0·38	0·49	0·00	0·44	1·71
No 5-methyltryptophan	8·5	3·98	3·90	0·55	0·23	0·56	0·08	0·72	2·14
No 5-methyltryptophan	45	11·6	10·5	2·27	0·06	1·68	1·05	2·55	7·61
5-Methyltryptophan added 4 min. before bacteriophage	45	2·90	2·78	0·39	0·30	0·45	0·05	0·42	1·61
5-Methyltryptophan added 9 min. after bacteriophage	45	7·6	7·2	1·46	0·04	1·13	0·66	1·76	5·05
			Experiment 2						
Infected in original growth medium containing tryptophan	45	8·9	8·1	2·01	0·09	0·79	0·97	2·14	6·00
Infected in original medium, centrifuged 5 min. later, washed and resuspended. No tryptophan	38 after resuspending	8·4	7·42	1·71	0·11	0·71	0·78	2·11	5·42
Washed before infecting. No tryptophan	45	3·8	3·17	0·51	0·52	0·39	0·00	0·64	2·06

decreased. It will be seen from Table 5 that the recoveries of the DNA bases on hydrolysis (55–75%) were lower than those of about 80% obtained by Hershey *et al.* (1953). The causes of the larger losses in the present experiments are not known.

DISCUSSION

Whilst Barner & Cohen (1954) found that bacteriophage T2 induces the synthesis of thymine in a thymine-requiring mutant of *Esch. coli*, the evidence in the present work is that the phage does not induce the synthesis of the amino acids and purines required by the six mutants which have been studied. Each of the four amino acid-requiring mutants has been found to require the appropriate amino acid (tyrosine, tryptophan, leucine or histidine) for an early stage in the development of bacteriophage following the infection of the host cell. Although the purine-requiring mutants require purines for the net synthesis of nucleic acids they do not require purines to support the early stages of bacteriophage development. Presumably, as in the normal infective cycle, the adenine and guanine in the nucleic acids of the host cell are utilized for the formation of bacteriophage nucleic acid (Koch, Putnam & Evans, 1952).

Washed cells of a nicotinamide-requiring mutant of *Esch. coli* (Gots' B-159) have been found to be capable of supporting phage formation in the mannitol medium containing no nicotinamide (K. Burton, unpublished). The latent period and burst size were similar to those obtained in the presence of nicotinamide or with the wild-type strain. This is consistent with the absence of any increase of the respiration rate following the infection with phage (Cohen & Anderson, 1946). It also indicates that the requirement for tryptophan cannot be due to its possible role as a precursor of nicotinamide.

The amino-acid requirements for phage development and also the inhibition by 5-methyltryptophan (Cohen & Fowler, 1947) show that the proteins of the host cell cannot be degraded to provide any appreciable amount of the required amino acids under conditions of amino acid deficiency. This is in agreement with the findings that protein synthesis and amino acid utilization continue during normal phage development until shortly before lysis (Cohen, 1948; Raff & Cohen, 1950) and that almost all the bacteriophage protein is synthesized from the constituents of the medium after infection (Siddiqi, Kozloff, Putnam & Evans, 1952).

If it is correct that the amino acid requirements studied in the present work are due to the dependence of bacteriophage development on the

synthesis of protein, it would follow that the first stage of the infective cycle necessitating protein synthesis occurs after the introduction of the DNA of the infecting phage into the bacterial cell. It precedes the decrease in the ultraviolet sensitivity, the conversion of bacterial DNA into DNA containing the characteristic viral pyrimidine (5-hydroxymethylcytosine) and the net synthesis of DNA. If no tyrosine or tryptophan is available during later stages of the phage development, the synthesis of bacteriophage DNA continues although no mature phage appear.

These phenomena suggest that the first proteins synthesized after infection include the enzymes and/or templates necessary for synthesizing the phage-specific DNA which differs from the bacterial DNA. Once the synthesis of this DNA has been initiated, it can continue independently of the synthesis of proteins containing tyrosine or tryptophan. This independence may be related to the fact that uninfected amino acid-requiring bacteria can synthesize nucleic acids in the absence of amino acids required for growth.

Recently, two groups of workers have been studying the action of chloramphenicol on T2-infected *Esch. coli* (Melechen; Rosenbaum, Halvorson, Preston & Levinthal, private communications). The drug appears to act similarly to 5-methyltryptophan in that it can, under suitable conditions, inhibit the synthesis of DNA if added before the infecting phage; if added 10 min. after the infection it allows DNA synthesis to continue but still prevents bacteriophage formation. In uninfected bacteria, chloramphenicol inhibits the synthesis of bacterial protein without inhibiting the synthesis of bacterial nucleic acid (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954).

The conclusions of this paper are related to the findings of Hershey, Garen, Fraser & Hudis (1954), who studied the incorporation of ^{35}S from inorganic sulphate into the protein of infected cells and the subsequent appearance of the tracer in the phage protein. Their results show that only about 10% of the sulphur incorporated by the cells during the first 5 min. after infection appears in the mature phage, whereas at later times 50–60% of the incorporated sulphur appears in the phage. Hence it is likely that the proteins which are required to be synthesized in order to initiate the synthesis of DNA do not themselves form part of the structure of the bacteriophage.

SUMMARY

1. By the use of amino acid-requiring mutants of *Escherichia coli* B as host cells, histidine, leucine, tryptophan and tyrosine have been shown to be required for the multiplication of T2 bacteriophage after the entry of the deoxyribonucleic acid (DNA) of the infecting phage into the host. Without the required amino acid the infected cells cannot develop the increased resistance to the action of ultraviolet light in suppressing the formation of bacteriophage.

2. The amino acid requirements for the synthesis of bacteriophage DNA were studied using two of the strains (tyrosine- or tryptophan-requiring). In the absence of the required amino acid at the time of infection, there was no net synthesis of DNA and no appearance of the characteristic T2 pyrimidine (5-hydroxymethylcytosine) in the DNA of the infected cells.

3. When the amino acid was present for the first 5 min. after infection and then removed by washing the infected cells, no mature bacteriophage appeared, but DNA (containing 5-hydroxymethylcytosine) was synthesized at about 80% of the rate which occurred when the amino acid was present.

4. Analogous results were obtained with the wild-type strain of *Esch. coli* B and an antimetabolite (5-methyltryptophan) which prevents the biosynthesis of tryptophan. When added 3 min. before the infecting bacteriophage there was no net synthesis of DNA and little 5-hydroxymethylcytosine appeared in the DNA of the infected cells. When 5-methyltryptophan was added 7 min. after the infecting bacteriophage, no mature bacteriophage was formed, but DNA containing 5-hydroxymethylcytosine was synthesized at about 60% of the normal rate.

5. It is concluded that, after infection of the host cell, there must be a synthesis of protein before the synthesis of the characteristic bacteriophage DNA is initiated. When these proteins have been synthesized, the synthesis of bacteriophage DNA is largely independent of the synthesis of proteins containing tyrosine or tryptophan, although further protein synthesis is necessary for bacteriophage formation.

6. Some experiments with purine-requiring mutants as host bacteria are also described. Purines were not required to be present in the growth medium during the early stages of bacteriophage development. Presumably the DNA of the host cell is utilized for the bacteriophage development.

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Chromatographic Studies on Corticotropin

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Adrenocorticotrophic hormone has been assayed and defined in several ways, but of late the term has been restricted to substances active in the ascorbic acid depletion test of Sayers, Sayers & Woodbury (1948). It is with factors active in this test by intravenous injection into hypophysectomized rats that this paper is concerned. Corticotropin A has been chemically characterized (White, 1953) after isolation from pig pituitaries and shown to possess activity. The designation A is to distinguish it from corticotropin B, isolated after treatment of concentrates with pepsin by Brink *et al.* (1952). As we, in agreement with Bell (1954), are reporting the presence of more than one active substance in such concentrates before hydrolysis, it is at present convenient to give these designations A_1, A_2 etc. The corticotropin B group may require similar subdivision.

Preliminary results (Dixon, Moore, Stack-Dunne & Young, 1951) suggested the use of ion-exchange chromatography with a carboxylic resin, because of the success of this method with the basic proteins ribonuclease and lysozyme (Hirs, Moore & Stein, 1953; Tallan & Stein, 1953). Some of our earlier results have been published, in particular the separation of the active material from a preparation of the protein hormone which had been thought to be the active substance. Extension of the method to concentrates gave better results than with crude materials (Dixon, Stack-Dunne & Young, 1953). Many other workers have since used ion-exchange chromatography with ACTH, and the chief successes of the method have been the isolation of corticotropin A (White & Fierce, 1953) and α-corticotropin (Li *et al.* 1954).

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METHODS

Materials

(1) *Pituitary powder.* Whole pig pituitaries were collected in acetone and dried with acetone after mincing. The pituitaries were removed within 1 hr. of death, and the final drying was within 10 days. Potency, 0·1–0·2 i.u./mg.

(2) *Crude corticotropin and oxycellulose-purified corticotropin (OPC preparation).* These were prepared from whole-pituitary powder by the methods of Payne, Raben & Astwood (1950) and Astwood, Raben, Payne & Grady (1951).

(3) *Fraction L (crude prolactin).* This was prepared by a modification of the method of Lyons (1937). The advantage of raising the acetone concentration during the extraction was retained by adding further acetone, before separating the solids, after extraction at a low acetone concentration. The preparation was carried out at 0–3°.

Pituitary powder (250 g.) was stirred with 1·5 l. of acetone, 0·94 l. of water and 70 ml. of 11·6 N-HCl (a final concentration of 60%, v/v, acetone) overnight. Acetone (2·5 l.) was added over 1 hr. with stirring (acetone concentration 80%, v/v), the mixture stirred for 15 min. and the supernatant separated by centrifuging. The solids were re-extracted with 1·25 l. of 80% (v/v) acetone for 0·5–1 hr. and separated. The combined extracts (about 4·8 l.) were mixed with 9·3 l. of acetone and allowed to stand overnight. The precipitated solids were dried with acetone. Weight yield 7–10%. ACTH potency 1–2 i.u./mg.

(4) *Fraction OL.* This preparation was made at room temperature. Fraction L (20 g.) was dissolved in at least 250 ml. of 0·1 N acetic acid and centrifuged if necessary. Sufficient Amberlite IR-4B acetate was stirred with the solution to remove the chloride originally present in L, and the solution filtered. The solution was treated with 2·0 g. of oxycellulose in a total volume of 1 l. of 0·1 N acetic acid, and the preparation continued as described by Astwood *et al.* (1951). Weight yield, 2·0–2·5%. Potency, 15–40 i.u./mg.

Chromatography

The carboxylic resin Amberlite IRC-50 in the ground form XE-64 was used after the cleaning and preparation described by Hirs *et al.* (1953). By alternate stirring and settling two fractions were selected. The one used for most of the work was of such particle size as to give a flow rate of about 5 ml./cm.²/hr. under gravity alone when packed in a column of 30–40 cm. height. The finer fraction required a pressure of 15–25 cm. Hg to maintain half this rate of flow. The buffer used for most of the work was 0·2 M in phosphate and 0·3 N in Na (pH 6·7). The sodium salt of the resin was equilibrated with the buffer, when it was in suspension in 0·2 M phosphate, by the addition of 0·2 M-Na_2HPO_4 or 0·2 M-NaH_2PO_4 until the pH of a sample of filtrate from the suspension was within 0·01 unit of that of the buffer. Care is needed (cf. Hirs *et al.* 1953) to ensure that equilibrium has been attained. Phenol (0·2%, v/v) was added to the buffer to act as a preservative.

No antioxidant has been found necessary for the chromatography of concentrates at the relatively high loads described. Thiodiglycol was previously used (Dixon *et al.* 1951) in the chromatography of crude extracts of ACTH, but one sample of thiodiglycol was found to destroy the biological activity.

Columns were prepared and operated according to the methods of Hirs *et al.* (1953). A flow rate of 2–4 ml./cm.²/hr. was used, and a load of 5–20 mg./cm.² of oxycellulose-purified concentrates, or about a third of this for the rechromatography of single components. The sample was applied for chromatography in a volume of buffer corresponding to 1·0–1·5 ml./cm.² of the column. Fractions, each of about 1·5 ml./cm.², were collected.

Expression of chromatographic results. The concentration of solute in the eluate was determined by the photoelectric ninhydrin methods of Moore & Stein (1948, 1954). This was plotted against the volume of eluate, V, from the application of the sample, corrected to what it would have been for a 100 ml. column resin bed. $V_{100} = 100 V_{obs.}/v$, where v = resin bed volume. The fraction size was checked by weighing the tubes and the resin bed volume accurately determined.

In elution chromatography it can be shown that $E - F = bK$, where E is the retention volume (the volume of effluent from the application of the sample to the emergence of the solute in maximum concentration), F is the volume of liquid in the bed, b is the volume of stationary phase, and K is the partition coefficient of the solute between the stationary phase and the liquid, in favour of the stationary phase. Also $N = E(E-F)/S^2$, where N is the number of theoretical plates in the column, and S is the standard deviation of the distribution curve representing the concentration of solute in the effluent as measured in terms of effluent volume. These expressions may be derived as follows (Stack-Dunne, 1954):

If the column is considered as a series of 'plates' where the concentrations of solute in the mobile and static phases are brought into equilibrium with each other in each plate separately, and the mobile phase in each plate is then transferred quantitatively to the next one along, the operation is analogous to that of a countercurrent machine operated according to the single withdrawal scheme of Craig (1950), and the same mathematical treatment can be applied.

The symbols already defined are used, and also the following: T = the number of transfers up to the point of emergence of the solute in maximum concentration, and p = the fraction of the solute in the mobile phase of any plate at equilibrium. Then

$$(1-p)/p = Kb/F \qquad (1)$$

since the factor b/F corrects the partition coefficient for the different volumes of the phases.

After t transfers the distribution of solute in the plates is given by the terms of the binomial expansion of

$$(p + [1-p])^t,$$

and of these the $(pt+1)$th term is the largest. The solute therefore travels at p times the rate of the solvent.

Thus after T transfers the solute emerges from the column at maximum concentration (at effluent volume E), where $pT = N$, and

$$p = F/E = N/T. \qquad (2)$$

By combining eqns. 1 and 2 we obtain $E - F = bK$. This relationship has also been derived by Duin (1952).

The standard deviation of the distribution in the tubes, i.e. $(p + [1-p])^t$, in terms of number of tubes, is given by

$$S_T = \sqrt{[tp(1-p)]}. \qquad (3)$$

The distribution of the solute in the effluent is a reflexion of the distribution on the column. When the peak concentration emerges, $t = T$, and it can be assumed that because N is large, the width of the distribution relative to N will be small, and that all the emergent material has been subjected to T transfers. In fact, the leading portions will have had less than, and the trailing portions more than, T transfers; these effects tend to cancel out.

If the peak of the distribution is moved the distance of one plate on the column, an effluent volume of E/N must emerge, and the contents of one plate will be in this volume. Hence

$$S_T = SN/E \quad \text{or} \quad S = ES_T/N \qquad (4)$$

This equation may be rewritten, by using eqn. 3 and putting $t = T$, as

$$S = \frac{E}{N}\sqrt{[Tp(1-p)]},$$

from eqn. 2 and eliminating T and p this becomes:

$$N = \frac{E(E-F)}{S^2}.$$

This equation is somewhat similar to one derived by Mayer & Tompkins (1947). It can be applied to determine the resolving power from the plot of any elution chromatogram, with or without knowledge of the units in which the effluent is measured. The relations between the terms here used and the conventional R values are:

$$R = v/E \quad \text{and} \quad R_F = F/E.$$

Isolation of products after chromatography (desalting). (1) By ion exchange. The solution was adjusted to pH 3·0 and allowed to run through a 5 cm.-high column of the same fraction of Amberlite IRC-50 (XE-64) that was used in chromatography. The resin was in its acid form. The load of solution was 10 ml./cm.² and the flow rate 10 ml./cm.²/hr. The column was washed with 1 ml./cm.² of 0·1 N acetic acid and the hormone displaced with 1 M-NH_3 solution. The fractions close to the break-through of alkali were combined and dried while frozen. The whole process was carried out at 0–5° and the columns were cooled with jackets of ice and water. This method has been found to cause modification of the A_1 component and its use has therefore been abandoned.

Modifications of this method may be used for purifying crude material. Extracts of pituitaries in 0·2 N-HCl may be adjusted with sodium acetate to pH 3 and run through columns of Amberlite IRC-50 (beads or ground form). The active material is retained and inactive matter may be washed out with 0·1 N acetic acid. Elution with M ammonium acetate solution of pH 9·5 displaces further inactive material first, and the active material shortly before the alkaline break-through. This method has not been developed further. The active material was not adsorbed by oxycellulose from the medium of ammonium acetate after acidification with HCl (at pH 3·0 or 4·5).

Aqueous pyridine (25%, v/v) did not displace the active material from a desalting column, in agreement with the findings of Richter, Ayer, Bazemore, Brink & Folkers (1953). A solution of pyridine (30%, v/v) and acetic acid (4%, v/v) rapidly displaced active material without modification after the resin had been washed with pyridine. Inorganic material, which had evidently been adsorbed together with the hormone, was, however, simultaneously displaced.

(2) By phenol extraction. The principle of White, Fierce & Lesh (1951) has been used as follows:

Reagents: Phenol, redistilled under reduced pressure, equilibrated with 0·01 N-HCl; 0·01 N-HCl saturated with phenol; 0·01 N-HCl; peroxide-free wet ether. The fraction was adjusted to pH 3·5 with conc. HCl. This acidified solution (100 ml.) was extracted with 30 ml. of phenol and twice more with 20 ml. of phenol. Each phenol extract was washed six times with 10 ml. of 0·01 N-HCl (saturated with phenol) by passing it in turn through six separating funnels. The phenol extracts were combined (60 ml.) and to them were added 60 ml. of 0·01 N-HCl and 320 ml. of ether. After equilibration, the aqueous phase was passed through two washes, each of 60 ml. of ether, and the three ether phases extracted twice in turn with 60 ml. of 0·01 N-HCl. The combined aqueous phases were treated with Amberlite IR-4B acetate or Amberlite IRA-400 bicarbonate to remove the HCl and were re-extracted with 60 ml. of ether. The clean resin had been washed shortly before use with water, ethanol, ether, ethanol, water and, for the acetate, dilute (0·1%) acetic acid. Part of the dissolved ether was removed by boiling the solution under reduced pressure before freezing and drying while frozen. Thiodiglycol is not removed in this process.

Ionophoresis on paper

The method of Gordon, Gross, O'Connor & Pitt-Rivers (1952) has been used. Following these authors, veronal buffer (0·05 M) of pH 8·8 was used and Whatman no. 3 filter paper. The paper was dipped in the buffer and blotted. Samples of 50–400 μg. (usually 200) were applied in 10–20 μl. of buffer or water. A potential of about 2·5 v/cm. and a time of 16 hr. were found to give optimum separation. Staining with bromophenol blue (Durrum, 1950) and spraying with 0·2% ninhydrin in *n*-butanol were used for the detection of spots. Samples were applied close to the equilibrium positions of their components (where migration was balanced by flow, which was due to capillary replacement of evaporation in addition to electro-osmosis) to attain the sharpest spots (cf. Durrum, 1950).

Pepsin hydrolysis

Samples were incubated at a concentration of 1 mg./ml. in 0·01 N-HCl with 2% of their weight of crystalline pepsin (Armour), at 37°. Incubation times of 1·5, 3 and 24 hr. were studied. There was little inactivation under these conditions in 3 hr. The hydrolysate was heated in 2–3 min. to 80°, maintained at this temperature for 5 min. and cooled in 2–3 min. Excess of Amberlite IR-4B acetate was added to remove chloride and the solution filtered and dried while frozen. Weight yield, about 90%.

Bio-assay

The ascorbic acid depletion method of Sayers *et al.* (1948) was used with minor modifications (Stack-Dunne, 1953). Wistar albino rats of 200–350 g. weight were used. The ascorbic acid was determined by electrometric titration.

Amino acid analysis

A sample of 5 mg. was boiled under reflux for 24 hr. in a mixture of 5 ml. 10 N-HCl and 5 ml. of 90% (v/v) formic acid. The hydrolysate was analysed by chromatography on a column of the sulphonic resin Dowex-50, with N-, 2·5 N- and 4 N-HCl as eluents (Stein & Moore, 1949). The fractions of eluate were evaporated to dryness and allowed to react with ninhydrin under the conditions specified by Troll & Cannan (1953).

RESULTS

Chromatographic patterns. Chromatography of OPC preparations (oxycellulose concentrates of acetic acid extracts of pituitaries) showed resolution of both activity and ninhydrin-reacting material into two main peaks (Fig. 1) and a minor peak. There was, in addition, an unretarded peak of ninhydrin-reacting material which had small but definite activity. A similar pattern was found with preparation OL (an oxycellulose concentrate of an acid–acetone extract of pituitaries) (Fig. 2).

The positions of the peaks agreed well between chromatograms when columns of 0·8–1·0 cm.

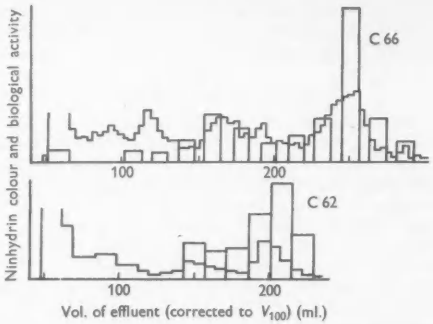

Fig. 1. Chromatography of oxycellulose concentrates of glacial acetic acid extracts of pituitaries. Continuous line: ninhydrin colour. Blocks: biological activity. Column C66 was 3·51 cm.^{2} × 39·5 cm. and the load was 188 mg. of an OPC preparation made by using the usual nitrogen tetroxide oxycellulose with 11% of CO_2H. Column C62 was 0·68 cm.^{2} × 40·0 cm. and the load was 10 mg. of an OPC preparation made by using periodate chlorite oxycellulose with 8% of CO_2H.

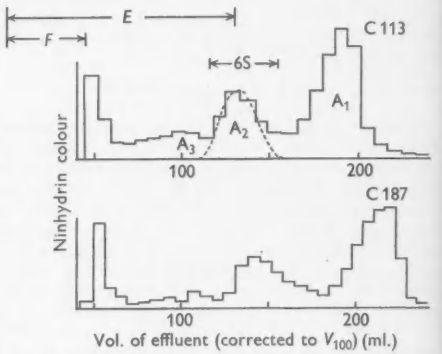

Fig. 2. Chromatography of an oxycellulose concentrate of corticotropin (preparation OL). Analytical column C113 was 0·6 cm.^{2} × 34·2 cm. and the load was 15 mg. Preparative column C187 was 13·4 cm.^{2} × 29·2 cm. and the load was 205 mg.

Table 1. *Effects of changes in the chromatographic system on the retention volumes of corticotropin peaks*

Columns of 0·8–1·0 cm. diameter and 20–40 cm. height (except last)

Sodium phosphate buffer: Normality of Na†	pH	*E* values (ml./100 ml. resin bed) for: A_1	A_2	A_3	Notes	Column no.
		190	129	98	OL sample	C113
		192	136	103	A_1 sample modified by ion-exchange desalting	C119
		188	133	103	Same sample after heating in glacial acetic acid	C120
		182	128	—	OL sample; 1% (v/v) benzyl alcohol added to buffer	C122
0·3	6·70	188	131	104	OL sample; 0·5% BRIJ 35† added to buffer	C123
		186	132	99	A_1 sample modified by ion-exchange desalting	C125
		183	130	—	OL sample; zinc acetate added to sample	C173
		192	135	102	OPC sample	C174
	Means	188	132	102		
0·3	~~5·69~~ 6·53	226	156	120	OL sample; phosphate 0·207 M	C148
0·3	6·79	174	125	87	OL sample; phosphate 0·194 M	C147
0·35	6·70	130	104	—	OL sample; phosphate 0·232 M	C142
0·25	6·70	362	216	140	OL sample; phosphate 0·168 M	C145
0·366	7·42	77	Not resolved		OL sample; phosphate 0·2 M; system suitable for some corticotropin B components; Na_2HPO_4/NaH_2PO_4 = 5 (molar ratio)	C193
0·3	6·70	283	187	—	A_1 sample modified by ion-exchange desalting	C177*
		283	190	139	OL sample	C186*
0·1	9·0	600	—	—	OL sample; system of White & Fierce (1953) (bicarbonate)	C201
0·103	8·5	239	143	—	OPC sample; column in collaboration with E. B. Astwood, M. S. Raben & I. Rosenburg for study of intermedin and adipokinin; column 1·5 cm. diam. × 70 cm.	D3‡

* Finer resin sample used.
† A polyoxyethylene lauryl alcohol (Atlas Powder Co., Wilmington, Delaware).
‡ Using Amberlite IRC-50 (XE-97).

CORRIGENDUM

Chromatographic studies on corticotropin

By H. B. F. Dixon and M. P. Stack-Dunne

Volume 61 (1955)

p. 486, Table 1, Col. 2, second figure down

For 5·69 *read* 6·59

diameter were used (Table 1, top section), but preparative columns of 2 or 4 cm. in diameter showed more variable and larger retention volumes, and this is illustrated in Table 2 (cf. Figs. 1 and 2). The peaks are named A_1, A_2 and A_3 as shown in Fig. 2. After peak A_1 further active material could be displaced from the resin with N ammonia solution. This material is analogous to the types II and III ACTH of White & Fierce (1953).

A modification of the OL preparation was made, designed to shorten or make milder every step used after the acetone drying of the glands. Half the quantity of hydrochloric acid was used in the extraction and the final pH was 2·1, in place of the usual value of 1·5–1·7. The product was isolated and dried on the same day instead of being set aside overnight. In the treatment with oxycellulose the whole process was performed at 0–3° and the elution with hydrochloric acid was shortened to a total of 2 hr. Chromatography showed the typical pattern for OL and typical weight yields of the peaks were obtained (C209 in Table 3). It is therefore unlikely that any interconversion of peaks occurred during the preparation of OL from acetone-dried glands.

Table 2. *Comparison of retention volumes of corticotropin peaks on wide and narrow columns*

Sodium phosphate buffer, 0·3N-Na, pH 6·70 (0·2M phosphate). Retentions in ml./100 ml. resin bed. ±S.D. between columns. Analytical columns 0·8–1·0 cm. in diameter, 20–40 cm. in height; 8 columns; various samples (Table 1). Preparative columns 3·9–4·2 cm. in diameter, 29–34 cm. in height; 5 columns; OL samples.

Peak	Retention volumes	
	Analytical	Preparative
A_1	188±4	212±12
A_2	132±3	141± 5
A_3	102±3	105± 5

Table 3. *Potencies of fractions isolated from OL by chromatography*

Fractions A_1 and A_2 consist of the resolved peaks A_1 and A_2. Fraction A_3 contains the imperfectly resolved peak A_3 and fraction A_4 signifies the unretarded material. Potencies are expressed in i.u./mg. followed in brackets by the fiducial limits for $P = 0{\cdot}95$.

Potency of sample	Fraction	Weight yield (%)	Potency	Activity yield (%)	Remarks
18 (11–30)	A_4	5·9	1·0 (0·3–3)	0·35	C115. Desalted by ion exchange
	A_3	4·7	—	—	
	A_2	10·9	17 (12–23)	10·6	
	A_1	14·8	30 (22–40)	26	
		36			
18 (11–30)	A_4	6·8	—	—	C127. Desalted by ion exchange. (Before desalting A_1 had potency of about 36)
	A_3	5·6	—	—	
	A_2	11·9	—	—	
	A_1	21·4	20 (8–53)	26	
		46			
17 (9–30)	A_4	6·5	—	—	C161. Desalted by ion exchange
	A_3	2·4	17 (8–36)	3	
	A_2	10·0	24 (11–49)	14	
	A_1	18·5	39 (23–67)	44	
		38		61	
17 (9–30)	A_4	2·2	—	—	C164. Desalted by ion exchange. Column run at 0°
	A_3	2·0	—	—	
	A_2	7·9	—	—	
	A_1	13	29 (11–73)	22	
		25			
33 (19–56)	A_4	Negligible	—	—	C182. Phenol desalted
	A_3	7	—	—	
	A_2	13	23 (9–60)	9	
	A_1	26	42 (21–86)	33	
		46		42	
33 (19–56)	A_3	5	—	—	C187. Phenol desalted
	A_2	26	—	—	
	A_1	25	35 (24–50)	—	
		56			
	A_3	3	—	—	C209. Phenol desalted. OL rapidly prepared
	A_2	13	—	—	
	A_1	21	32 (15–68)	—	

When the OL preparation, instead of being made from acetone-dried pituitaries, was made from frozen pituitaries, or pituitaries which had been allowed to autolyse for one day, a different chromatographic pattern was obtained. The A_2 peak was smaller, only 10% of the A_1 peak in ninhydrin reaction, in place of the usual 30–50%. A similar result was obtained when acid–acetone was used for drying the glands. Fig. 3 shows the comparison of OL preparations made from one batch of frozen glands, 25 g. of which (about 100 glands) were dried for 6 days in 250 ml. of acetone, and another 25 g. of which were dried in 250 ml. of acetone containing 1·5 ml. of 10N hydrochloric acid. Half the usual quantity of hydrochloric acid was added in the subsequent extraction with 60% acetone of the batch previously treated with acid. These results suggest that the hormone is more predominantly in one form in frozen glands, glands dried with acid acetone and glands allowed limited autolysis than it is in the acetone-dried glands used for the greater part of this work. The small A_2 peak of the acid–acetone-dried preparation possessed biological potency comparable with A_1 with respect to ninhydrin colour.

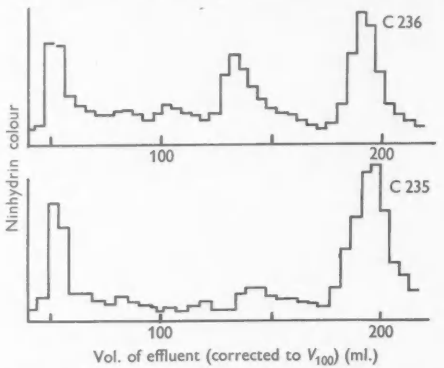

Fig. 3. Comparison of oxycellulose concentrates from glands dried with acetone and with acid–acetone. Column C236 was 0·6 cm.^{2} × 39·8 cm. and the load was 6·2 mg. of OL from pituitaries dried with acetone alone. Column C235 was 0·6 cm.^{2} × 39·8 cm. and the load was 5·3 mg. of OL from pituitaries dried with acid–acetone.

On rechromatography the isolated components ran true with respect to retention volume (Fig. 4). The A_1 peak shown (C184) had been desalted by extraction into phenol, since ion-exchange desalting modified this material. The A_2 sample (C224) was derived from a preparative chromatogram of OL and the tails of its peak probably represent heterogeneity; an A_2 peak without such tails is shown in Fig. 6. The A_3 peak (C144 of Fig. 4) was derived from a chromatogram of the A_2 and A_3 peaks of several columns.

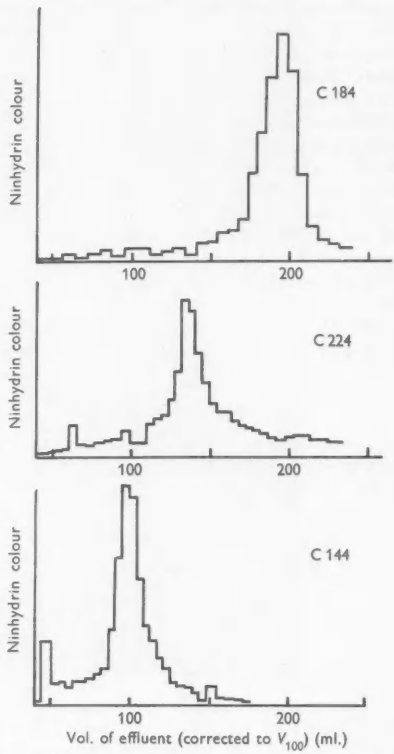

Fig. 4. Rechromatography of A_1, A_2 and A_3 materials. Column C184 was 0·68 cm.^{2} × 29·2 cm. and the load was 3·3 mg. of A_1 material phenol-desalted from a preparative chromatogram. Column C224 was 0·6 cm.^{2} × 40·6 cm. and the load was 1·8 mg. of A_2 material phenol-desalted from a preparative chromatogram. Column C144 was 0·68 cm.^{2} × 25·6 cm. and the load was 3·2 mg. of A_3 material from a second chromatogram of several A_2 and A_3 fractions (desalted by ion exchange).

Modification occurred when A_1 material was desalted by ion exchange. Chromatography after such desalting showed the appearance of A_2 and A_3 peaks (the latter in only small amounts). Different amounts appeared in the different samples desalted. A sample of A_1 from a preparative chromatogram of an OL sample was divided and parts were desalted by the two different methods. Rechromatography (Fig. 5) showed production of A_2 in the sample desalted by ion exchange, but not in that extracted into phenol. A second ion-exchange

desalting of the A_1 that had survived the first produced further conversion into A_2, as shown in C135 of Fig. 5, showing that there was no part of the A_1 which resisted the change. The assays shown in Fig. 5 suggested that no change of biological activity accompanied the modification.

After standing overnight at pH 11·3 in buffer (Na_2CO_3, 0·28 M; Na_3PO_4, 0·043 M; Na_2HPO_4, 0·007 M), A_1 material was completely converted into A_2 as C225 (Fig. 6) shows. A sample of 5·2 mg. of A_1 was used, and in the first 5 hr. 1–1·5 μM of volatile base was given off and the evolution was nearly complete. This result was consistent with the loss of one group of amide ammonia from a substance of molecular weight 4000–5000.

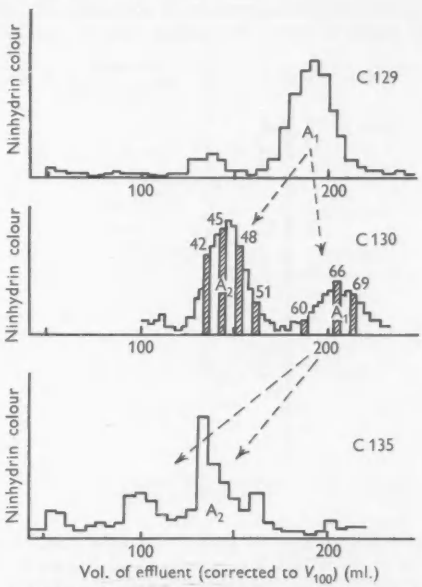

Fig. 5. Modification of A_1 peak on desalting by ion exchange. Comparison of columns C129 and C130 shows the modification. C135 shows that the residual A_1 of C130 can also undergo the change. C129 was 0·68 cm.^{2} × 30·1 cm. and the load was 10 mg. of A_1 peak from a preparative column desalted by the phenol method. C130 was 3·5 cm.^{2} × 29·7 cm. and the load 56 mg. of A_1 peak from a preparative column desalted by ion exchange. Assays (Sayers *et al.* 1948) on marked tubes are shown below. The fractions were tested at a dose equal with respect to their ninhydrin colour.

Tube no.	Ascorbic acid change (mg./100 g.)	S.E.M.	No. of rats
42	−97	15	4
45	−88	11	5
48	−94	16	4
51	−77	9	4
60	−102	12	4
66	−90	13	5
69	−67	7	4

Conclusion: no significant change in biological activity occurred on conversion of A_1 peak into A_2. C135 was 0·68 cm.^{2} × 41·0 cm. and the load 16 mg. of the A_1 peak from C130 desalted by ion exchange.

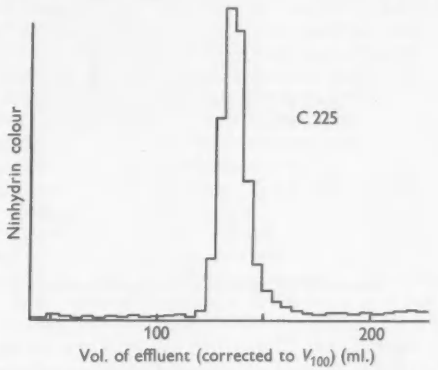

Fig. 6. Chromatography of corticotropin A_2 produced by alkaline treatment of A_1 material. Column C225 was 0·6 cm.^{2} × 40·6 cm. and the load about 3 mg. of phenol-desalted product after corticotropin A_1 had stood for 15 hr. in solution in buffer of pH 11·3.

The asymmetry of the A_1 peak (e.g. Fig. 2, C187) suggested that two partially resolved components might be present. Accordingly, the forward and backward parts of this peak from a preparative chromatogram were separately desalted and rechromatographed. Fig. 7 shows the results with the preparative column and the analytical columns. As usual the retention volumes were larger with the wider column, but agreement of position of the peak is shown between the two analytical columns, despite the failure of the fraction collector to give regular fractions in C191. This result suggests that the asymmetry of the peak was not due to inhomogeneity of A_1.

Characterization of peak materials. Table 3 gives the potencies of the peak materials isolated from chromatograms. Potencies of 30–40 i.u./mg. have been achieved for corticotropin A_1.

There was no evidence of heterogeneity of A_1 material on electrophoresis at pH 8·2 in glycine–NaCl buffer (mobility $-1{\cdot}3 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ at 18–19°; $I = 0{\cdot}1$; glycine 0·0086 M; NaCl 0·0986 M), or at pH 5·1 in acetate–NaCl buffer (mobility $+0{\cdot}4 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ at 0°; $I = 0{\cdot}2$; acetate 0·0265 M; NaCl 0·09 M). This is consistent with an isoelectric point between these pH values, as Li *et al.* (1954) have reported for sheep α-corticotropin.

Ultracentrifuging at pH values of 7·6 and 2·7 revealed only one component, with sedimentation values of 0·85 and 0·72 Svedberg unit respectively (corrected to water at 20°).

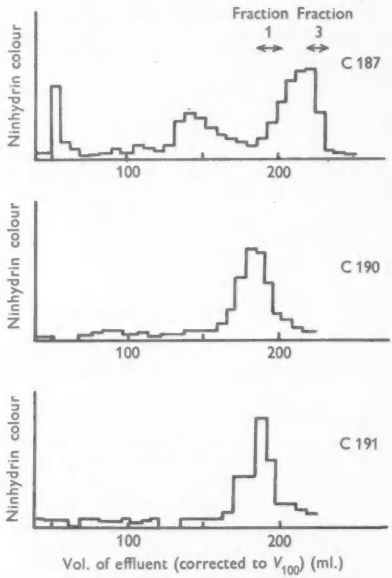

Fig. 7. Chromatographic comparison of leading and trailing edges of A_1 peak. The positions of the A_1 peaks in columns C190 and C191 agree. As usual, the peaks are less retarded than in the wider column C187. Preparative column C187 was 13·4 cm.^{2} × 29·2 cm. and the load 205 mg. of OL fraction. Column C190 was 0·6 cm.^{2} × 26·2 cm. and the load 1·48 mg. of phenol-desalted fraction 1 from C187 (above). Recovery of ninhydrin colour, 81%. Column C191 was 0·6 cm.^{2} × 29·8 cm. and the load 1·36 mg. of phenol-desalted fraction 3 from C187 (above). Recovery of ninhydrin colour, 78%.

Ionophoresis on paper by the method of Gordon *et al.* (1952) was suitable for characterizing certain of the concentrates. Fig. 8 shows the patterns given by the different desalted fractions from a chromatogram. These fractions were desalted by ion exchange, and consequently the A_1 peak contained A_2 material. Other samples have shown only the spot which moves least from the origin. The chromatographically unretarded fraction is shown to be heterogeneous, and the A_2 and A_3 fractions are not distinguished. The spot corresponding with that from A_1 which is present in the A_2 fraction has been repeatedly found in A_2 samples from preparative chromatograms of OL that by chromatographic criteria are free from A_1, and it is thought to represent not merely contamination with A_1 material.

Samples of A_1 material were exposed to the different conditions of ion-exchange desalting and analysed by paper electrophoresis in order to discover which of the conditions involved had caused the modification. Contact with N ammonia, with or without the presence of Amberlite IRC-50, was found to effect the change into A_2 material.

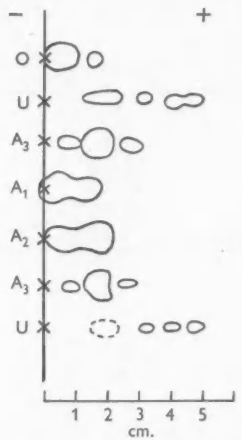

Fig. 8. Ionophoresis on paper of fractions (desalted by ion exchange) from a preparative chromatogram of OL. Samples (200 μg.) applied in 10 μl. Ionophoresis in 0·05 M veronal buffer, pH 8·8. O, OL sample; U, unretarded fraction; A_1, A_2 and A_3, peak fractions.

The material was isolated by drying while frozen after most of the ammonia had been evaporated off. Only the faster-moving spot was present on electrophoresis. Other alkaline conditions were found to cause the change, such as standing in solution adjusted to pH 12 with sodium hydroxide. The reagent was removed with Amberlite IR-120 before freeze-drying. Ethylamine (25%) produced the change, but not 50% pyridine (after being set aside overnight at room temperature). Only partial change occurred in 2 hr. at 0° in N ammonia. After alkali had thus been found to effect the change the alteration was confirmed chromatographically as described above (Fig. 6). In this case too only the faster-moving spot was visible after analysis of the sample by paper electrophoresis.

A preliminary study of the products of peptic hydrolysis of corticotropins A_1 and A_2 has been

made, and the results are illustrated in Fig. 9. Families of more basic materials have been produced from both, and comparison (EC 58) shows that the products from A_1 are more basic (moved further towards the cathode) than those from the less basic A_2. The former are termed members of the corticotropin B_1 family and the latter members of the B_2 family. At least two of the products from A_1 were converted by standing in ammonia solution into products with the same mobility as those from A_2 (Fig. 9, EC 60). This suggests that the property of being altered by alkali to a less basic substance survived the peptic hydrolysis. If amide loss is the change concerned these results may be explained by the structure put foward by Bell (1954). These conditions of peptic hydrolysis (see Methods) caused little or no inactivation.

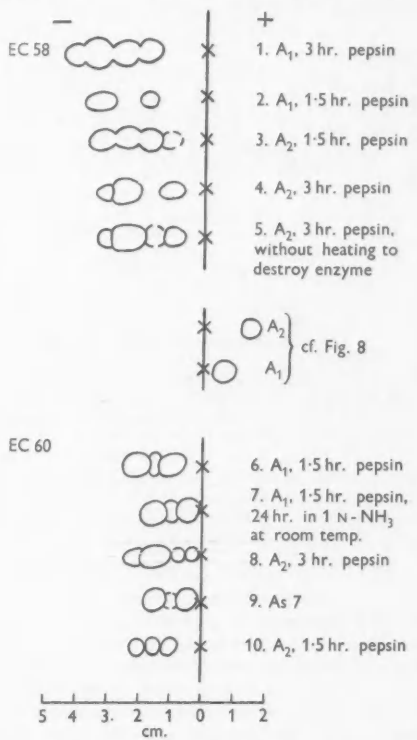
−
+
EC 58
1. A1, 3 hr. pepsin
2. A1, 1·5 hr. pepsin
3. A2, 1·5 hr. pepsin
4. A2, 3 hr. pepsin
5. A2, 3 hr. pepsin, without heating to destroy enzyme
A2
A1
cf. Fig. 8
EC 60
6. A1, 1·5 hr. pepsin
7. A1, 1·5 hr. pepsin, 24 hr. in 1 N-NH3 at room temp.
8. A2, 3 hr. pepsin
9. As 7
10. A2, 1·5 hr. pepsin
5 4 3 2 1 0 1 2
cm.

Fig. 9. Ionophoresis on paper of pepsin-treated materials (see Methods), in 0·05 M veronal buffer, pH 8·8. Samples of 200 μg. Sample 7 shows the change of B_1 components into B_2 components, analogous to the change of A_1 into A_2.

Modifications of the chromatographic system. Increase and decrease of the pH of chromatography, with a constant sodium-ion concentration in the buffer, respectively decreased and increased the retention volumes. The results of this and of other changes in the system are presented in Table 1. This change was expected, since increase of pH should neutralize the positive charges on the solute molecules. Another result was that increase of sodium-ion concentration decreased the elution volumes, and decrease of concentration similarly raised the volumes. This is explained by competition between solute and sodium ions for the adsorbent. The change in $(E-F)$ and hence of the partition coefficient was greater than inversely proportional to the cation concentration, as was also found for cytochrome *c* by Boardman & Partridge (1953).

The use of a finer sample of the resin greatly increased the elution volumes (Table 1). This effect could be explained if only a layer on the exterior of the particles, or fissures close to the outside, were available for the retention of substances of relatively high molecular weight. In this case 'b' would be less than $(v-F)$, and decrease of particle size would, through the increase of surface, increase the value of 'b', the effective volume of adsorbent. This explanation is consistent with the observation that the factor by which $(E-F)$ is increased is the same for peaks A_1, A_2 and A_3. The unretarded peak emerged at $V_{100}=50$, so that the value of 50 for F was unaltered. This excluded the possibility that more resin had been packed into the same bed volume.

The effects of increasing the pH and decreasing the sodium-ion concentration will tend to counteract each other in passing to the bicarbonate system of White & Fierce (1953) (Table 1). The difference with the sample of Amberlite IRC-50 (XE-97) in the bicarbonate system is probably largely due to a difference in particle size.

The use of a veronal buffer of pH 8·0 was partially successful, but some modification of the A_1 peak occurred during chromatography.

Resolving power of chromatograms. Application of the formula given for N shows that the preparative columns of 2–4 cm. diameter gave a resolution of about 5 plates/cm. [HETP (height of equivalent theoretical plate) = 2 mm.] for components A_1 and A_2. The analytical columns of 0·8–1·0 cm. diameter showed a resolution of about 10 plates/cm. (HETP = 1 mm.). The finer batch of resin showed a significantly greater resolution of about 40–100 plates/cm. (HETP = 0·1–0·25 mm.) (Figs. 10, 12). There was no evidence of resolution of the A_1 peak into further components in such columns of 1000–4000 plates. The mean particle diameter was 30–40 μ. in the coarser fraction of resin and 10–20 μ. in the finer.

Load on chromatographic columns. When the load on chromatograms was below 5 mg./cm.^{2} of OL, unsatisfactory patterns were obtained. Fig. 10 shows two such examples. The resolution appears decreased and the peaks spread backwards. It is possible that the lower limit of satisfactory load is reached earlier in cases such as these, where the peaks are more retarded (in one case owing to finer resin and in the other owing to decreased cation concentration).

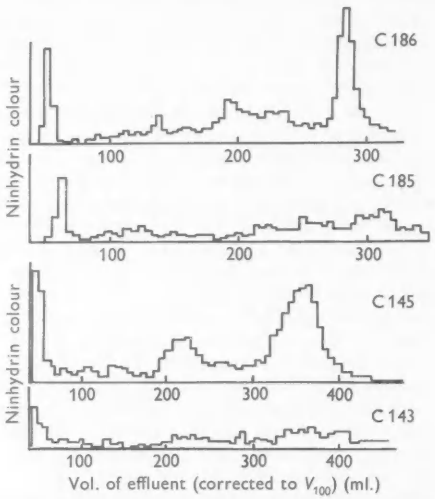

Fig. 10. Poor resolution exhibited by chromatograms at low loads of active material (OL samples). The columns had the following dimensions and loads: C186, 0·68 cm.^{2} × 38·3 cm. (load, 8·1 mg.); C185, 0·68 cm.^{2} × 39·4 cm. (load, 3·08 mg.); C145, 0·86 cm.^{2} × 17·0 cm. (load, 9·2 mg.); C143, 0·86 cm.^{2} × 17·2 cm. (load, 2·3 mg.). Columns C186 and C185 were of the finest resin sample (Na^+, 0·3N; pH, 6·70); columns C145 and C143 were of normal resin (Na^+, 0·25N; pH, 6·70).

A greater decrease of load prevents analysis by the ninhydrin reaction, but the sensitive bio-assay may be used to examine the effluent. When 250 μg. of OL were put on columns of 0·95 cm.^{2}, a greater decrease of resolution was observed, with increased retardation of the activity as well as spreading. This is shown in Fig. 11. This might have been due to the existence in the resin of some easily saturated sites which differed from the rest of the resin in affinity for the active substances. To test this possibility a sample of resin was treated with salmine sulphate (0·5 mg./ml. of resin bed, added in solution to a stirred suspension of the resin in the buffer) and a chromatograph run at low load with this resin. The ambiguous result is shown in C180 in Fig. 11.

This failure of the system at low loads contrasts with the behaviour of ribonuclease (Hirs *et al.* 1953). In that study, however, the load was somewhat higher in the successful chromatography of the enzyme from acid extracts of pancreas. The present failure explains the poor resolution in the chromatograms of crude extracts which has been previously reported (Dixon *et al.* 1951, 1953).

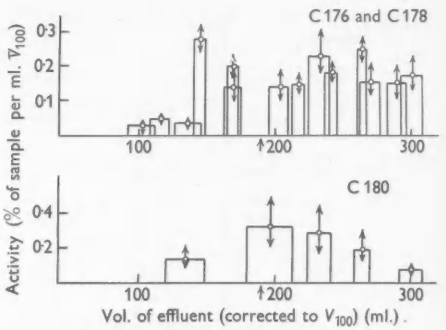

Fig. 11. Poor resolution exhibited by chromatograms at very low loads of active material (OL samples). Arrows ↔ mark ± S.E.M. → shows normal position of A_1. Data from columns C176 and C178 are pooled. C176 was 0·95 cm.^{2} × 22·5 cm. and C178 was 0·95 cm.^{2} × 24·3 cm. The recovery of activity was about 40%. C180 was 0·6 cm.^{2} × 27·4 cm. and the resin had been treated with salmine (see text). The recovery of activity was about 70%. The load on each column was 0·25 mg.

Phenol desalting. The method is satisfactory for the peak material as discussed above. An OL sample was submitted to the procedure, and some of it was lost in the aqueous phase (not extracted by phenol) and some in the ether phase (extracted together with phenol by ether), but appreciable amounts of peak material were not lost. After dissolving and desalting, the OL material recovered was chromatographed. The ninhydrin recovery of the chromatogram was 70% in place of the usual 40–50%, and this is probably due to the loss of components in the desalting which are retained by the resin on chromatography. The unretarded peak was also decreased. When the method was used with unretarded material from OL chromatograms little was recovered, and its initial presence stabilized phenol–water emulsions.

Chromatography of materials inactivated by treatment with hydrogen peroxide. A solution of OL in phosphate buffer was inactivated by adding 8% (v/v) of '100 volume' hydrogen peroxide and allowing 30 min. for reaction. The hydrogen

peroxide was decomposed by adding a solution of catalase, and a sample of the material was chromatographed. Over 99 % of the activity was destroyed by this treatment. In two cases the A_1 peak showed retention volumes of 181 and 182 (C 214, 215) in contrast with volumes of 195 and 197 (C 217, 222) obtained with the same batch of resin with the original sample of OL. In order to show separation of the active and inactivated substances, a solution of corticotropin A_1 was treated with hydrogen peroxide, and further corticotropin A_1 dissolved after the peroxide had been destroyed with catalase. With the normal resin batch a clear-cut separation was not obtained, although the shape of the peak suggested two components, but the separation was satisfactory when the finer sample of resin was used as shown in Fig. 12. Assay showed that the faster peak was inactive and the slower active.

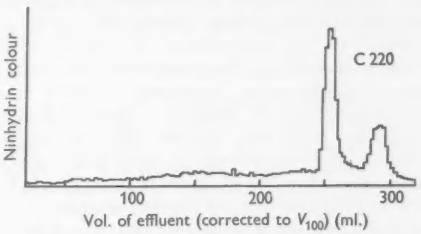

Fig. 12. Separation of active A_1 from A_1 inactivated by treatment with H_2O_2. Column C220 (very fine resin sample) was 0·68 cm.^{2} × 44·1 cm. 6·8 mg. of A_1 was dissolved in 1·25 ml. of buffer; 0·1 ml. of 100-vol. H_2O_2 was added; after 30 min. 0·02 ml. of catalase solution (0·006 M in catalase haemin) was added and 6·5 mg. of A_1; 1·00 ml. on column.

Reactivation of material inactivated by peroxide was not achieved at room temperature with H_2S or 4 % aqueous potassium borohydride (which did not inactivate active material). In confirmation of the results of Dedman, Farmer & Morris (1955), inactivated corticotropin A_1 (25 μg./ml.) was found to be reactivated by heating with cysteine (0·06 M) at pH values of 3·4 and 1·9 at 74° for 18 hr.

Amino acid analysis. Only one sample of corticotropin A_1 was analysed and one check of recoveries made, so that reliable analytical figures were not found for all the amino acids. The following numbers of amino acids gave the best fit to the results, assuming a molecular weight of 4600. Glycine 3 (3·04), alanine 3 (2·96), aspartic acid 2 (1·76), leucine 2 (2·08) and histidine 1 (1·01). No cystine or isoleucine was present. Analysis of another sample after treatment with 1-fluoro-2:4-dinitrobenzene (FDNB) by the method of Sanger (1945) confirmed these figures (except for histidine, which reacted with the FDNB). Difficulties were met with other amino acids, but the following values were suggested by the results: serine 1–3, glutamic acid 4–5, valine 2–3, tyrosine 1–2, lysine 3–4, arginine 2–3, phenylalanine 2–4 and methionine present. The ultraviolet absorption spectrum in alkali (Beaven & Holiday, 1952) suggested: tyrosine 2, tryptophan 1.

Amide groups were determined by hydrolysis at 100° in 2 N-HCl for 3 hr. and estimation of the ammonia colorimetrically after microdiffusion in Conway units. The amide nitrogen was 1·9 % of the total nitrogen, suggesting that one amide group was present.

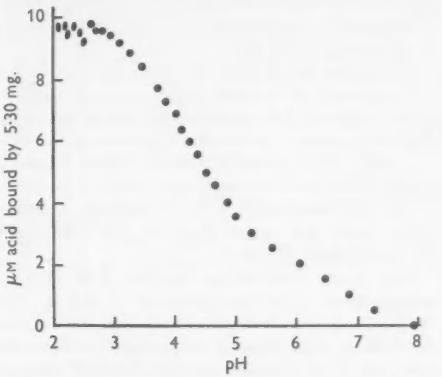

Fig. 13. Titration curve of corticotropin A_1 in 0·7 M-NaCl. Corticotropin A_1 (5·30 mg.) in 1·00 ml. of water, 0·10 ml. of 0·1 N-NaOH and 1·10 ml. of 1·4 M-NaCl titrated with 0·05 N-HCl in 0·65 M-NaCl. A blank titration was used to give corrections. 0·7 M-NaCl was used to minimize the discrepancy between the measured pH and $-\log_{10}[H^+]$ (Danckwerts, 1952).

The titration curve (Fig. 13) shows the binding of eight μequiv. of acid by 5·3 mg. corticotropin A_1 between pH values of 2·5 and 6. This is consistent with the presence of about seven free carboxyl groups in corticotropin A_1, assuming that its molecular weight is 4600 as suggested by the formula of Bell (1954) as well as by the analysis reported here. From its method of preparation the sample was unlikely to contain acetate.

DISCUSSION

Gradient elution or a discontinuous change in eluent are useful for the chromatographic separation of substances of widely differing affinities for the absorbent. Higher resolution is to be expected in elution chromatography under conditions where

the eluent is in equilibrium with the absorbent, since the substance chromatographed will move at constant speed down the column. Under these conditions there is no fear that a single sharp peak of several substances of greatly differing affinity for the resin will be displaced by a sudden change of eluent, such as may occur when gradient elution is attempted with resins of high buffering power. Amberlite IRC-50 buffers less at higher pH values, such as those used by White & Fierce (1953) or by Li *et al.* (1954), but modification of the A_1 component is a possible danger under these conditions. The satisfactory resolution achieved in the present experiments may be attributed to the equilibrium conditions and low pH.

The chromatographic results, especially those with columns of very fine resin and high resolving power, suggest that corticotropin A_1 is an essentially homogeneous material. This is supported by the claim of White (1953) that a similar chromatographic product contained only 15% of impurity, for the published plot of his chromatogram shows lower resolution than that of the preparative columns described here.

The main observation against this claim of homogeneity is the low potency of the products (Table 3). The values previously reported include 100–150 i.u./mg. (White, 1953) and 80–100 i.u./mg. (Bell, 1954) in contrast with the present values of up to 50 i.u./mg. The standardizations originally made against Armour preparation 84–85 H have been checked against U.S.P. corticotropin and Armour preparation 60–61. Apart from a possible systematic error in the assay of potent materials, these low potencies might be explained by the presence of considerable amounts of inactive material. In view of the rigour of the methods used for separation, such material would most probably be inactivated ACTH rather than chance contaminant. It would have to differ from the active substance less than the material produced by treatment with hydrogen peroxide, since no peak corresponding with this material was observed on chromatography of normal concentrates (Fig. 10). A decrease of elution volume on Amberlite IRC-50 on treatment with peroxide has also been reported for ribonuclease (Ledoux, 1954), but in that case is referable to the oxidation of thiol groups.

The consistently lower potency of isolated corticotropin A_2 in comparison with A_1 (Table 3) may not be significant. The A_2 peak from chromatograms of OL is probably heterogeneous (electrophoretically, Fig. 8, and chromatographically, Fig. 10, C 186) and some contaminant may be inactive. The equal potency of the A_2 produced from A_1 (Fig. 5) is not contradictory to this. No evidence has been obtained about the relative potency of purified A_3, since the conditions of the preparative columns are not suitable for obtaining this component in a pure state. Its peak clearly overlaps a considerable ninhydrin-reacting background.

The preliminary amino acid analysis for corticotropin A_1, with its points of agreement with that of White (1953), in particular the absence of cystine and isoleucine [cf. also Bell (1954) and Li *et al.* (1954) for sheep material, and Brink *et al.* (1953) for corticotropin B] bears out the suggestion from the chromatographic data that we are concerned with the same material, corticotropin A. It is probable from its predominance in the extracts that corticotropin A_1 is also the β-corticotropin of Bell (1954).

The nature of the change of corticotropin A_1 to other products is not explained. Loss of amide ammonia is almost certainly a factor. But the production of A_3 and the fact that A_2 prepared chromatographically from OL shows two spots on paper ionophoresis (in contrast with the single spot shown by A_2 prepared by alkaline treatment of A_1) suggest that other changes may be involved. Since only one amide group is present, other forms of unmasking of acid groups or masking of basic groups (e.g. by acyl migration) would be needed to explain the change unless amino acid residues were lost by the molecule.

Acetone-drying causes partial modification of A_1 material, but, even with the conditions of collection which at present seem best, some active A_2 material exists in the glands. Increasing the time of autolysis did not greatly increase the amount of the A_2 material, so the hour which elapses between the death of the pig and removal of the gland is probably not responsible for its presence.

SUMMARY

1. Corticotropin concentrates have been chromatographed on a carboxylic resin in sodium phosphate buffer. Active components have been resolved. The elution volumes are reproducible on chromatography of the isolated components.

2. Methods have been used for isolating the active components from salt solutions, such as the buffers used in chromatography, which depend on the adsorption of the material on a carboxylic resin at pH 3 or on extraction of the material into phenol.

3. The main active component has been designated corticotropin A_1. Other components (corticotropins A_2 and A_3) are present which are less retarded on the resin columns and their amount may be increased under certain conditions. Alkali converts corticotropin A_1 into A_2.

4. Corticotropins A_1 and A_2 can be distinguished by ionophoresis on paper.

5. When corticotropin A_1 is treated with pepsin, inactivation does not occur, but a group of new substances, termed collectively corticotropin B_1, is produced. The B_1 components are separable by ionophoresis on paper. A different family (B_2) is produced from corticotropin A_2.

6. Alkali converts the corticotropin B_1 family into the B_2 family, a change analogous to that of A_1 to A_2.

7. The effects of the cation concentration and pH of the buffer and of the particle size of the resin on the chromatographic elution volumes are described (Table 1).

8. From the method of isolation it seems that corticotropin A_1 is identical with the corticotropin A previously described by other workers.

We wish to express our gratitude to the Nuffield Foundation for their generous support of this work. We are greatly indebted to Professor F. G. Young, F.R.S., for his interest, support and advice throughout the study, and to Dr Stanford Moore for his continued interest and advice since the period of our collaboration. We are also indebted to Miss Marjorie Thomas for her skilled assistance throughout, and to Miss Diane Cope and Miss Angela Baston for assistance during large parts of the work. We are grateful to Mr M. W. Rees and to Dr F. Sanger, F.R.S., for many helpful discussions, and to the Armour Laboratories, the Rohm and Haas Co., the Shirley Cotton Research Institute, the Tennessee Eastman Co. and to Dr E. F. Hartree for the gift of samples. One of us (H.B.F.D.) held the Benn W. Levy Studentship from Cambridge University and received a research training grant from the Medical Research Council of Ireland and a grant from the H. E. Durham Fund of King's College, Cambridge, for parts of the work. We are grateful to Mr B. R. Slater for the electrophoretic analysis, to Mr B. Boon for the ultracentrifuge data and to Mr P. C. Reed for nelp in preparing the figures.

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Utilization of Glucose for the Synthesis of Protein in Chicken and Rat Liver and Rat Hepatoma *in vitro*

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Zamecnik, Loftfield, Stephenson & Steele (1951) showed that when slices of rat hepatoma were incubated with uniformly labelled [^{14}C]glucose the radioactivity of the tissue protein was 10–15 times that of normal liver tissue taken from the same animal and treated in a similar manner. Zamecnik Frantz, Loftfield & Stephenson (1948) had previously used [^{14}C]alanine in similar experiments in which they showed that there was a greater incorporation of radioactivity into the hepatoma than into the liver slices, although the differences were not as great as those found with [^{14}C]glucose. The high growth rate of malignant tissue as compared with its tissue of origin, and the possibility that tumour tissue may be able to utilize glucose more effectively for the synthesis of protein than normal tissue, are good reasons for the further investigation of glucose utilization for the synthesis of protein.

Rabinovitz, Olson & Greenberg (1954) have shown that an exchange reaction can take place *in vitro* between preformed protein and the amino acid pool. Thus under these conditions the incorporation of radioactive amino acid into protein does not necessarily imply that complete protein synthesis has occurred. It was desirable, therefore, to study the transfer of radioactivity from [^{14}C]-glucose to a protein, the synthesis of which could be followed. Peters & Anfinsen (1950) have described such a system in which the amount of serum albumin formed by chick-liver slices is estimated by an immunological method. However, it seemed improbable that any extensive synthesis of albumin would take place in rat hepatoma, so that it was decided first to compare the incorporation of ^{14}C into both the serum albumin and a soluble protein fraction after incubation of chick-liver slices with [^{14}C]alanine and [^{14}C]glucose. Alanine was chosen as the amino acid, since it is known to be formed from glucose. It would then be possible to determine the ratio of the specific radioactivity of alanine in the albumin, of which there had been a net synthesis during incubation, and in the soluble protein fraction, after incubation of liver slices with [^{14}C]alanine and [^{14}C]glucose respectively. If the ratio of specific radioactivities were found to be the same with the two substrates then it would seem probable that the transfer of ^{14}C from [^{14}C]glucose to the soluble liver-protein fraction is a reflexion of the utilization of glucose for protein synthesis. The experiments reported show that this is in fact the case.

Zamecnik *et al.* (1951) showed that the amount of glycogen present in hepatoma slices was very small compared with that present in liver slices. Thus if [^{14}C]glucose is added to liver slices the dilution of [^{14}C]glucose through the breakdown of glycogen is much greater in the liver than in the hepatoma slices. Zamecnik *et al.* (1951) overcame this difficulty by starving the animals over a prolonged period (6 days), after which the amount of glycogen in the liver was negligible. However, Wyshak & Chaikoff (1953) have shown that the metabolism of glucose in the liver is greatly reduced in rats which have been starved for 3 days or more. Thus it seemed undesirable to starve the rats in the present experiments. The difficulty arising from the dilution of [^{14}C]glucose was, therefore, overcome by measuring the specific radioactivity of the glucose during the course of the experiment. It is shown that when the specific radioactivity of the glucose present in the medium in which the hepatoma and liver slices are incubated is approximately the same, the radioactivity of the tissue protein from the hepatoma is very much higher than that from the liver slices.

MATERIALS AND METHODS

Animals. Chickens were of the Brown Leghorn strain, bred at the A.R.C. Poultry Centre, Edinburgh.

Rats were Wistar albinos highly inbred at this Institute. They were fed on rat-cube diet (No. 86, Rowett Institute, Aberdeen, Scotland).

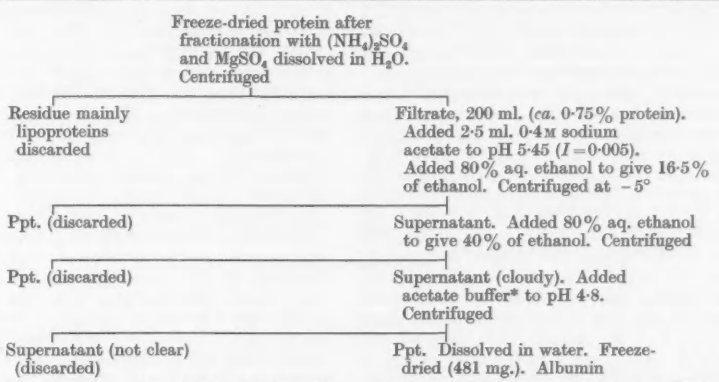
Production of hepatomas. Male rats (160–180 g.) were kept on a diet containing 0·06% *p*-dimethylaminoazobenzene. The diet, which was fed *ad libitum*, was prepared as described by Kline, Miller, Rusch & Baumann (1946). The casein (Lactic; unextracted) and the salt mixture (DL6) were obtained from Glaxo Laboratories, Greenford, Middlesex. The maize oil was obtained from the British Oil and Cake Mills, Albion Wharf, Erith, London, S.E. Vitamins A and D were supplied in the form of Adexolin (Glaxo Laboratories) at the rate of 1 drop/kg. of diet. After 10 weeks on the diet the animals were subjected to laparotomy and placed on a normal diet. They were maintained

on the normal diet for at least 2 weeks before being used. After this treatment most of the animals had liver tumours. Some of the livers were affected in only one lobe, whereas others were widely infiltrated with tumour. When the metabolism of 'normal' and hepatoma tissue was to be compared, the livers of several rats were used. The 'normal' liver slices were obtained from the unaffected lobes of livers, which contained only small amounts of tumour tissue.

Radioactive substances. Uniformly labelled D-[^{14}C]-glucose (100 μc $\equiv$ 1·64 mg.), L-[^{14}C]alanine (100 μc $\equiv$ 5·2 mg.) and [^{14}C]glycine (100 μc $\equiv$ 8·6 mg.) were supplied by the Radiochemical Centre, Amersham.

thus prepared. This was examined in a Tiselius electrophoresis apparatus (A. Hilger and Co., London) in veronal and acetate buffer (I = 0·1) at a concentration of 1–2% protein.

Preparation of antisera against chick albumin. An alum-precipitate of the chick albumin (389 mg.) was prepared by the method of Proom (1943). The precipitate was suspended in 25 ml. M/15, pH 6·7, phosphate buffer containing 1:10 000 Thiomersalate (British Drug Houses Ltd.) and homogenized in a glass homogenizer. Rabbits were then given nine intravenous injections of the suspension by the ear vein over 3 weeks as follows: 2 of 0·2 ml., 2 of 0·4 ml., 5 of 0·8 ml. Ten days after the last injection the rabbits


Freeze-dried protein after fractionation with $(NH_4)_2SO_4$ and $MgSO_4$ dissolved in H_2O. Centrifuged
Residue mainly lipoproteins discarded
Filtrate, 200 ml. (ca. 0·75% protein). Added 2·5 ml. 0·4M sodium acetate to pH 5·45 (I = 0·005). Added 80% aq. ethanol to give 16·5% of ethanol. Centrifuged at −5°
Ppt. (discarded)
Supernatant. Added 80% aq. ethanol to give 40% of ethanol. Centrifuged
Ppt. (discarded)
Supernatant (cloudy). Added acetate buffer* to pH 4·8. Centrifuged
Supernatant (not clear) (discarded)
Ppt. Dissolved in water. Freeze-dried (481 mg.). Albumin

* Buffer 5 ml. 10N acetic acid, 2·5 ml. 4M sodium acetate, 10·5 ml. of ethanol. Water to 25 ml.

Fig. 1. Purification of chick serum albumin by ethanol fractionation.

Purification of chick serum albumin. Although Peters & Anfinsen (1950) described the use of antiserum for estimating the amount of albumin formed by chick-liver slices, they did not give any adequate details for the preparation of chick albumin. A brief description of the method used is therefore given.

The chickens were starved overnight and then killed by decapitation. Blood was collected and allowed to clot, and the serum separated by centrifugation. The serum was treated with $MgSO_4$ according to the method of Popják & McCarthy (1946). After removal of the precipitate by filtration the $MgSO_4$ was removed from the filtrate by dialysis and the solution was treated with 0·5 saturated $(NH_4)_2SO_4$ (31 g./100 ml.). After removal of the precipitate the filtrate was saturated with $(NH_4)_2SO_4$, the precipitate collected by filtration, dissolved in water and dialysed against distilled water until free of $(NH_4)_2SO_4$. Examination of this solution by filter-paper electrophoresis in veronal buffer, pH 8·6, I = 0·05 (Flynn & de Mayo, 1951), showed it to contain only traces of globulin in addition to albumin. The purification of albumin was then completed with ethanol by a modification of Method 6, of Cohn *et al.* (1946). Details of the method used are given in Fig. 1. The fractionation described applies to the serum obtained from three hens (each about 2 kg. body weight) which gave 70 ml. of serum in all. The composition of the various fractions was followed throughout the procedure by filter-paper electrophoresis. Altogether 1·03 g. of albumin were

were killed and serum was collected. The serum was fractionated by the addition of 2 vol. of 27% (w/v) Na_2SO_4 dissolved in sodium phosphate buffer, pH 7·8 (I = 0·1), to produce a final concentration of 18% (w/v). The precipitated globulins were redissolved in water and reprecipitated at 16% (w/v) Na_2SO_4, dialysed and freeze-dried. The amount of antibody in the rabbit serum was estimated and found to average 10 mg./ml. of serum. A test for the homogeneity of the antiserum was carried out according to Oudin (1948). This showed that in addition to the main component three components are present in very small amounts.

Incubation procedure for tissue slices. Slices were cut and suspended in a bicarbonate medium as described by Peters & Anfinsen (1950). After a preliminary washing of the slices in the medium (150 ml. of medium for 6 g. of slices) at room temperature, to reduce the amount of albumin present, they were drained on filter paper and divided into the appropriate number of batches. Each weighed batch of slices was incubated in 10 ml. of the same medium, which contained any additions of radioactive glucose or amino acid required, at 37° in a gas phase of $O_2 + CO_2$ (95:5).

In the experiments with chicks, the liver slices were obtained from animals 6–12 weeks old and about 2 g. of slices were used in each incubation flask. In the experiments involving rat liver and hepatoma only about 1 g. of tissue was used in each flask.

Microdetermination of serum albumin in liver and hepatoma slices. The method used is based on that of Peters & Anfinsen (1950). Preliminary tests showed that in order to obtain a satisfactory calibration curve over the range 5–60 μg. of albumin, it was necessary to use 3 mg. of freeze-dried antiserum. The procedure as applied to the estimation of albumin in liver and hepatoma slices was as follows.

After incubation of the slices at 37° the flasks are cooled on ice. The slices and medium are then homogenized in a Potter-type glass homogenizer and the tissue suspensions are centrifuged at 2° for 20 min. at 15 000 *g*. The supernatant, which is not clear, is diluted with an equal vol. of 0·9% NaCl containing 1:10000 Thiomersalate and frozen overnight. This procedure appears to cause denaturation of the lipoproteins, for the supernatant is usually completely clear after a second centrifugation. For the accurate estimation of albumin it is essential that this be so.

For each estimation 0·3 ml. of a 1% solution of freeze-dried antiserum in 0·9% NaCl, which has been completely cleared by centrifuging, is used. Sufficient of the supernatant from the tissue homogenates is used to give 10–60 μg. albumin. For comparison tubes containing similar quantities of albumin are set up as standards. The volume of each tube is made up to 1·5 ml. with 0·9% NaCl, and tubes are incubated at 37° for 40 min. and then set aside overnight at 2°. The precipitate is spun down at 2°, washed twice with 0·9% NaCl at 2° and then dissolved in 1·5 ml. 0·1 M-Na_2CO_3. After complete solution of the protein precipitate has been ensured the tubes are again centrifuged to spin down any insoluble material. The optical density of the clear solutions is read at 280 mμ. in a spectrophotometer with 0·5 cm. quartz cells. Determinations were made at two different concentrations on each tissue supernatant and the results were averaged.

Large-scale precipitation of albumins with antisera. When sufficient albumin was required for determination of radioactivity, an excess of antiserum was added to the extract containing the albumin. After incubation the precipitated albumin was centrifuged and the supernatant tested to ensure excess of antiserum. The precipitated albumin was then washed with 0·9% NaCl at 2° and then with ethanol, ethanol–ether–$CHCl_3$ (2:2:1, v/v) and finally acetone.

Measurement of radioactivity

Protein. Trichloroacetic acid (TCA) precipitates of the proteins were treated as described by Zamecnik *et al.* (1951). The dried protein was then plated either on 1 cm.^{2} polythene disks or on 0·3 cm.^{2} Perspex disks and counted at infinite thickness as described previously (Campbell & Work, 1952).

Specific activity of alanine in tissue proteins. After hydrolysis of the protein (5–10 mg.) in a sealed tube with 5 N-HCl for 18 hr. at 110°, alanine was isolated by paper chromatography by using first phenol–water (4:1) and then pyridine–water (4:1), as described by Steinberg & Anfinsen (1952). Whatman no. 3 paper was used for chromatography. The paper was pretreated by washing first with N-Na_2CO_3, then with distilled water, 2 N-acetic acid and finally distilled water. Ordinary descending chromatographic procedures were used for this washing, each solvent being run for 24 hr. With paper thus treated the R_F of alanine when phenol was used as solvent was consistently less than that of the marker alanine. It was, therefore, necessary to determine the position of the alanine on these chromatograms by using absorption under ultraviolet light. The purity of the alanine obtained by these methods was checked using butanol–acetic acid–water (4:1:5) (Partridge, 1948) as solvent for a third chromatogram with Whatman no. 4 paper. The alanine obtained was dissolved in 10% aq. *iso*propanol and samples were placed on clean 2 cm.^{2} polythene disks. A 0·05% solution of the non-ionic detergent BRIJ 35 (Honeywill and Stein Ltd., 21 St James's Square, London) was added to each disk to effect a complete spread of the alanine solution. The disks were then dried in a vacuum desiccator over P_2O_5. The radioactivity of the disks was determined by means of a Geiger end-window counter. The amount of alanine on each disk was determined by reaction with ninhydrin, according to the method of Cocking & Yemm (1954). The reaction was carried out in 1 in.-diameter boiling tubes capped with funnels, in an ammonia-free room. The tubes were heated for 20 min., cooled for 5 min. and diluted with 5 ml. of 60% ethanol. The colour was read in a spectrophotometer at 570 mμ. in 1 cm. cells. The procedure gave consistently lower blanks than that described by Moore & Stein (1948) and was found to be convenient. A satisfactory standard curve for amounts of alanine between 5 and 45 μg. was obtained.

Specific activity of glucose in tissue homogenates. Samples of the supernatant from homogenates of liver and hepatoma slices were quickly freeze-dried and then thoroughly dried in a vacuum desiccator over P_2O_5. The residue was extracted with 5 ml. of dry pyridine at 100° for 10 min. as described by Malpress & Morrison (1949). The pyridine was removed from the extracts under reduced pressure and the residues dissolved in 1 ml. of water. The aqueous extract was chromatographed overnight on Whatman no. 4 paper which had been washed with acetic acid and ammonia as recommended by Isherwood & Hanes (1953). Butanol–acetic acid–water (4:1:5) (Partridge, 1948) was used as the solvent. The position of the radioactive glucose on the chromatogram was determined by autoradiography. The glucose was eluted from the paper with water and its radioactivity determined as described for alanine. The amount of glucose on each disk was determined by the method of Hagedorn & Jensen (1923*a*, *b*). Although this procedure did not provide completely pure glucose, since there was some contamination with phosphorus-containing substances, its extent was slight and did not differ appreciably for tumour and liver tissue.

RESULTS

Chick serum albumin

Apart from the work of Peters & Anfinsen (1950) there seems to be no description in the literature of the purification of chick serum albumin. Before the method described was used, the methanol-precipitation method of Pillemer & Hutchinson (1945), which works well for the preparation of human serum albumin, was tried without success. In Fig. 2 are shown the electrophoretic diagrams obtained when chick serum albumin (1%) was examined in veronal buffer at pH 8·53 and in acetate buffer at pH 4·50. It will be observed that under these conditions there is no evidence of a

second component. However, the result of the Oudin procedure (see Methods section) showed that the albumin contained at least three minor components.

Synthesis of serum albumin in chick-liver slices

Fig. 3 shows the results obtained when a standard solution of chick serum albumin was assayed by the antiserum method previously described. Since small differences in the incubation and centrifuging procedures were liable to affect the results it was found more satisfactory to run standard tubes in each assay. The main source of difficulty was the presence of fat in the tissue extracts. High-speed centrifuging was the most effective method of clearing extracts of fatty suspensions, and this was greatly facilitated by the denaturation of lipoproteins which takes place on freezing the extracts. The results obtained with the method as described were usually accurate to within $\pm 10\%$, although, for no apparent reason, isolated results were sometimes obtained which were well outside these limits.

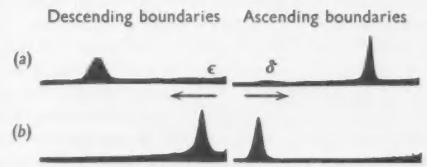


Fig. 2. Electrophoretic patterns of purified serum albumin. Each arrow indicates the position of the boundary at the start of the run and the direction of movement. Potential gradient, 4 v/cm. $I = 0{\cdot}1$, 1% protein. (*a*) Sodium veronal buffer, pH 8·53. Duration of run, 4 hr. 38 min. (*b*) Sodium acetate buffer, pH 4·50. Duration of run, 3 hr. 36 min.

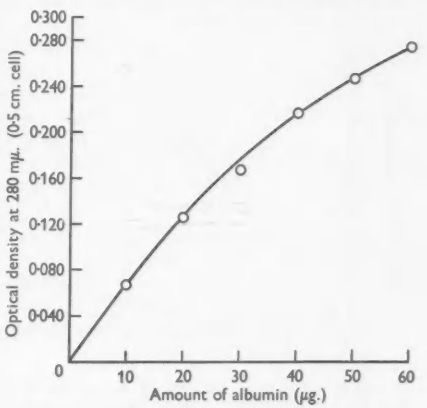


Fig. 3. Standard curve for estimation of chick serum albumin.

Altogether six experiments were carried out in which chick-liver slices were incubated in bicarbonate medium for different times and the amount of albumin in the medium plus slices was measured. In many of the experiments small quantities of radioactive amino acids or glucose were added to the slices before incubation and in these cases the radioactivity of the protein in the supernatant, which was precipitated by TCA, has been determined. The results of five such experiments are shown in Table 1. One other experiment has been omitted because the fatty nature of the liver made accurate estimation of the amount of albumin in the tissue extracts impossible.

Incorporation of radioactivity into various liver-protein fractions

In order to determine which liver-protein fractions incorporated the most radioactivity during the incubation of slices with uniformly labelled [^{14}C]glucose, the radioactivity of different protein fractions was determined. The results are shown in Table 2. The albumin, which was precipitated with antiserum, contained approximately ten times its weight of non-radioactive protein, since in the antibody–antigen precipitate the ratio of the two proteins is about 10:1. Thus the radioactivity of the albumin in the liver was very much higher than that of any of the other protein fractions tested.

Table 1. *Rate of synthesis of albumin in chick-liver slices*

Results are given in mg. albumin/g. wet tissue; percentage increase is shown in brackets. Radioactivity is expressed as counts/min./sq.cm. infinite thickness.

Expt. no.	Additions	0 hr. Albumin	0 hr. Counts	2 hr. Albumin	2 hr. Counts	4 hr. Albumin	4 hr. Counts
I	None	0·78	—	1·28 (64)	—	1·44 (85)	—
II	2 μc [^{14}C]glycine	1·66	0	1·98 (19)	118	2·40 (44)	321
III	2 μc [^{14}C]glucose	1·18	1	1·89 (60)	211	2·68 (127)	385
IV	20 μc [^{14}C]glucose*	1·36	—	1·74 (28)	—	2·02 (49)	2000
VI	5 μc [^{14}C]alanine*	0·78	—	1·01 (30)	—	1·72 (133)	1120

* Present only in the flask incubated for 4 hr.

Table 2. *Radioactivity of various protein fractions after incubation of chick-liver slices with [^{14}C]glucose for 2 and 4 hr.*

Radioactivity is expressed in counts/min./0·3 cm.^{2} infinite thickness.

Protein preparation	Count 2 hr.	Count 4 hr.
Homogenate of slices + medium; TCA ppt.	51	89
Supernatant of above; TCA ppt.	—	122
Supernatant of above; 0·5 saturated $(NH_4)_2SO_4$	79	—
Supernatant of above; saturated $(NH_4)_2SO_4$	70	—
Supernatant of above; albumin pptd. with antiserum	—	102

Estimation of specific radioactivity of alanine at infinite thinness

Fig. 4 shows the results obtained when the radioactivity of a standard solution of [^{14}C]alanine was estimated on 2 cm.^{2} polythene disks as described previously. The results show that over the range 10–70 μg. of alanine the amount of self-absorption is insignificant. Radioactive measurements on alanine isolated from protein were made on amounts of alanine which fell between these limits.

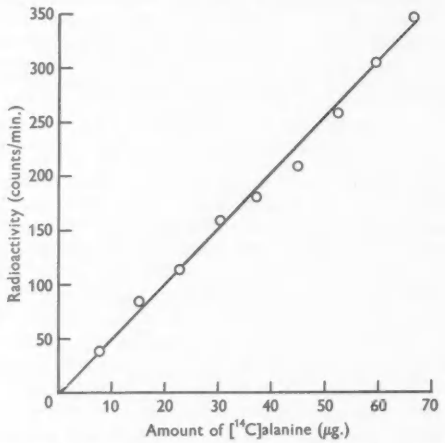


Fig. 4. Self-absorption curve for determination of specific radioactivity of [^{14}C]alanine.

As was previously explained (Methods section) the alanine isolated from protein hydrolysates by paper-partition chromatography was first assayed for radioactivity on polythene disks and then the amount of alanine on each disk was determined by interaction with ninhydrin. One of the dangers of such a method is that in eluting the alanine from the chromatogram some ninhydrin-reacting substance other than alanine may be eluted, even though the chromatography paper has undergone a careful washing procedure. On the basis that any such impurity in the [^{14}C]alanine would not be radioactive, the following method for checking the amount of such contamination of the alanine has been devised: the radioactivity of the alanine obtained from liver protein was determined on samples containing various amounts of alanine. The relationship between the amount of substance which reacts with ninhydrin and the radioactivity of the samples is shown in Fig. 5. The position where the graph cuts the base line must represent the amount of ninhydrin-reacting substance,

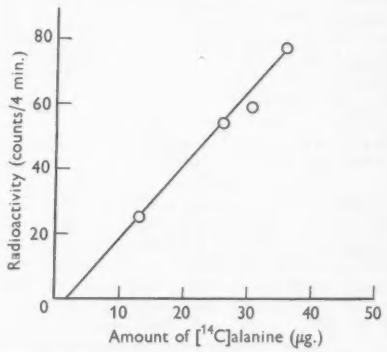


Fig. 5. Relation between amount of ninhydrin-reacting substance (expressed as alanine) and radioactivity of eluent from chromatogram.

which is not radioactive. It will be seen that the amount of such impurity is small compared with that of alanine. This method was considered more satisfactory than one involving the elution of control strips of the paper chromatogram. In triplicate determinations of the specific radioactivity of alanine the results obtained agreed to within $\pm 8\%$.

After chick-liver slices had been incubated with [^{14}C]glucose (Exp. IV, Table 1) for 4 hr. the slices and medium were homogenized and the suspension was centrifuged. One part of the supernatant

obtained was treated with antiserum and another with 10% TCA. Alanine was isolated from each of the precipitates and its specific radioactivity determined. A determination was made on similar precipitates obtained after incubation of chick-liver slices with [^{14}C]alanine (Exp. VI, Table 1). The results of these experiments are given in Table 3. As was previously observed the alanine obtained from the antiserum-precipitated albumin is diluted with the alanine present in the non-radioactive antiserum, but the alanine in each case will have been diluted to the same extent.

Determination of specific radioactivity of [^{14}C]*-glucose during incubation of normal liver slices and hepatoma slices and correlation with the radioactivity of the tissue protein*

The method used for measuring the radioactivity of the [^{14}C]glucose isolated from tissue extracts was similar to that used for [^{14}C]alanine. Since similar quantities of glucose and alanine were estimated, it has been assumed that the self-absorption of glucose in the radioactive assay is negligible, as was found for alanine. Since the Hagedorn & Jensen method used for the estimation of glucose is not as sensitive as the ninhydrin method used for the estimation of alanine, the accuracy of the assay for glucose is less than that for alanine. The accuracy of the determination of the specific activity of glucose is about $\pm 15\%$, which is sufficient for the present purpose of the experiments.

The results are shown (Table 4) of two experiments in which the specific radioactivity of the glucose in the medium and slices, and the radioactivity of the soluble protein obtained by precipitation with TCA, were estimated. It will be observed that the protein obtained from the tissue slices at time 0 hr. had negligible radioactivity. It was consistently found that the procedure adopted for the treatment of the tissue-protein precipitates ensured the removal of both free amino acids and glucose from the protein precipitates.

The amount of protein precipitated by the addition of TCA to the supernatant of the homogenates obtained from each flask was weighed, and was found to be closely similar for the hepatoma and liver tissue. Thus the amount of [^{14}C]glucose/g. tissue protein was the same for hepatoma and liver slices.

Preliminary experiments indicated that although the amount of glucose present in the liver slices during the incubation period was sufficient to enable the radioactivity of the glucose to be measured, this was not so in hepatoma slices. This was no doubt due to the small quantity of glycogen known to be present in hepatoma tissue compared with that in liver (Zamecnik *et al.* 1951). For this reason it was necessary to add inactive glucose to the hepatoma slices before the start of the incubation. The results shown in Table 4 suggest that in liver slices there is a rapid breakdown of glycogen leading to a lowering of the specific radioactivity of the glucose.

Table 3. *Specific radioactivity of alanine isolated from albumin and TCA-precipitated protein after incubation of chick-liver slices with* [^{14}C]*glucose and* [^{14}C]*alanine*

Radioactivity is expressed as counts/4 min./μM alanine.

	Incubation with	
	[^{14}C]glucose	[^{14}C]alanine
Albumin	185	980
TCA ppt.	183	848

DISCUSSION

Although Zamecnik *et al.* (1951) showed that under *in vitro* conditions hepatoma tissue utilized glucose more effectively for the synthesis of protein than did liver tissue, this result could not be confirmed *in vivo*. These authors were able to show that after feeding [^{14}C]glucose to rats the specific activity of the non-protein fraction of the hepatoma was less than that of the liver, whereas the radioactivity of the tissue proteins was not significantly different. This suggested a circulatory impairment of the hepatoma tissue, when compared with the normal liver tissue. They were later able to confirm this

Table 4. *Relationship between radioactivity of* [^{14}C]*glucose and tissue protein during incubation of rat-liver and hepatoma slices*

Results are expressed as counts/min./mg. glucose and counts/min./0·3 cm.^{2} protein at infinite thickness. Each incubation flask contained approx. 1 g. of tissue slices together with 0·033 mg. of [^{14}C]glucose in Expt. I and 0·013 mg. of [^{14}C]glucose in Expt. II. In addition, 10 mg. of inactive glucose was added to each flask containing the hepatoma slices.

		Liver			Hepatoma		
		0 hr.	2 hr.	4 hr.	0 hr.	2 hr.	4 hr.
Expt. I	Glucose	42 350	12 350	10 350	26 800	—	17 400
	Protein	2·5	19	33	3	—	327
Expt. II	Glucose	12 005	4 605	3 560	7 688	4 144	1 545
	Protein	0	9	21	0	61	105

after injection of [^{14}C]alanine. However, as Gaitonde & Richter (1955) have recently observed in brain tissue, a failure to incorporate an injected radioactive amino acid into a tissue protein does not imply a low rate of synthesis of the proteins in that tissue; a knowledge of the radioactivity of the free amino acid is necessary before any deductions may be drawn.

The purpose of the experiments described was to compare the utilization of glucose for the synthesis of protein in hepatoma and normal rat liver. However, it was not possible to follow the synthesis of a specific protein in the hepatoma tissue, and a mixture of the soluble proteins had to be used. It was desirable therefore that a comparison should be made between the transfer of ^{14}C from [^{14}C]glucose into a specific protein, of which there was a net synthesis in liver tissue, with the transfer of ^{14}C to a mixture of soluble liver proteins. Preliminary experiments (Table 2) indicated that such a mixture of soluble protein contained more radioactivity after incubation of liver slices with [^{14}C]-glucose than did the whole liver protein.

The experiments on chick-liver slices (Table 1), in which the amount of albumin was estimated during the course of incubation, leave little doubt that a net synthesis of albumin does take place under these conditions and thus confirms the results of Peters & Anfinsen (1950). The incorporation of [^{14}C]alanine into the albumin in Experiment VI suggests that the increase in albumin is real and that it is not merely being released from the tissue during the incubation period. However, the possibility of an exchange between the amino acid and the preformed protein, as mentioned in the Introduction, cannot be excluded.

The specific radioactivity of alanine in the albumin obtained after incubation of chick-liver slices with [^{14}C]glucose (Expt. IV, Table 1) under conditions of net albumin synthesis is compared with that in the soluble protein fraction in Table 3. Since the alanine in the albumin will have been diluted by that in the non-radioactive antiserum used in the precipitation of the albumin, the alanine in the albumin has a much higher specific radioactivity than that in the soluble protein fraction. In the utilization of glucose for protein synthesis the glucose must be metabolized to amino acids, which in turn are converted into protein. Since little is known about the distribution of the intracellular enzymes in these two reactions it was necessary to determine the specific radioactivity of an amino acid, known to be formed from glucose, in the two protein preparations after incubation with the radioactive amino acid. The amino acid chosen was alanine. The results of the specific radioactivity determinations are also shown in Table 3. It will be observed that within the experimental error of the methods used the ratio of the specific activities of alanine in the two protein preparations is the same in the two experiments. This demonstrated that alanine formed from glucose is relatively as available for the synthesis of albumin and the mixture of soluble proteins as is alanine added to the incubation medium.

The results of the experiments on rat-liver hepatoma may now be considered in the light of the results obtained with chick liver. Since the rat-liver and hepatoma slices were incubated with [^{14}C]glucose under the same conditions as the chick-liver slices we may assume that incorporation of radioactivity into the soluble protein fraction is a reflexion of the synthesis of protein. In the experiments for which the results are shown in Table 4, the radioactivity of the hepatoma protein is in each case higher than that of the corresponding liver protein. In the first experiment, the specific activity of the [^{14}C]glucose was greater in the hepatoma than in the liver, but the difference does not account for the tenfold difference in activity of the proteins. In the second experiment the activity of the glucose was always less in the hepatoma than in the liver, yet the hepatoma protein was five times as active as the liver protein. It is clear, therefore, that glucose is more effectively used for the synthesis of protein in hepatoma than normal liver tissue.

Three possible explanations of this result may be considered: First, hepatoma protein might contain a preponderance of those amino acids that are derived from glucose, compared with liver protein. Zamecnik, Frantz & Stephenson (1949) and Zamecnik & Frantz (1949) carried out an amino-acid assay of liver and hepatoma protein, but found no such differences. Secondly, it is possible that in hepatoma tissue there are mechanisms for synthesizing amino acids from glucose such as do not occur in liver tissue. Zamecnik *et al.* (1951) have examined this possibility, but proline was the only amino acid which was radioactive in the hepatoma protein and was not radioactive in the liver protein after incubation of the hepatoma and liver slices with [^{14}C]glucose. This could hardly account for the differences in activity of the proteins. Thus it must be concluded that the better utilization of glucose by hepatoma tissue for the synthesis of protein under *in vitro* conditions is due to a more rapid incorporation of amino acids into the hepatoma protein.

SUMMARY

1. The preparation of chick serum albumin is described. The preparation was homogeneous at pH 8·53 and 4·50 in the Tiselius electrophoresis

apparatus. However, an immunological method showed the presence of three additional minor components.

2. An antiserum, prepared by injection of chick serum albumin into rabbits, was used to demonstrate the net synthesis of serum albumin by chick-liver slices. The results of Peters & Anfinsen (1950) were thus confirmed.

3. The incorporation of radioactivity into serum albumin and a soluble protein fraction was studied after incubation of chick-liver slices with [^{14}C]glucose and [^{14}C]alanine respectively. The specific radioactivity of the alanine in each protein fraction was determined.

4. The incorporation of radioactivity into a soluble protein fraction of rat hepatoma and rat liver was studied after incubation of the tissue slices with [^{14}C]glucose. The specific radioactivity of the glucose during the incubation period was followed. It was thus shown that under these conditions there is a better utilization of glucose by hepatoma tissue for the synthesis of protein than there is by liver tissue.

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The Proteins of *Hevea brasiliensis* Latex

1. PROTEIN CONSTITUENTS OF FRESH LATEX SERUM

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Analytical studies (Altman, 1946) have shown that fresh unammoniated *Hevea* latex contains about 1% of protein, part of which is adsorbed at the surface of the rubber particles while the remainder is distributed between the aqueous serum phase of the latex and the lutoid bodies first described by Homans & van Gils (1948). Although it is generally recognized that the presence of protein in fresh latex is an important factor controlling its colloidal stability, further information on the concentration and properties of the individual protein components, and their distribution between the above three phases, is necessary for a better understanding of the colloidal behaviour of latex.

The present paper describes investigations on the proteins of fresh latex serum. Before 1942 the presence of three protein fractions in fresh serum had been demonstrated (Bishop, 1927; Kemp & Straitiff, 1940). Similar fractions were also obtained from ammonia-preserved latex, though the presence of ammonia modified the properties of the proteins in some respects (Kemp & Straitiff, 1940; Bondy & Freundlich, 1938). Later, a more detailed electrophoretic analysis of the proteins in unammoniated serum was carried out by Roe & Ewart (1942), who demonstrated the presence of seven electrophoretically distinct protein constituents.

The present paper confirms and extends many of Roe & Ewart's results. It is shown that the properties and composition of latex serum prepared by prolonged freezing of fresh latex differ in certain important respects from the serum obtained by centrifuging the latex.

EXPERIMENTAL

Materials

Freeze-dried serum. All experiments were carried out with latex from the same eighteen mixed clonal seedlings growing at the Rubber Research Institute of Malaya Experiment Station, and tapped regularly on a half-spiral, alternate-day tapping system. Latex collected in glass vessels surrounded by a mixture of ice and salt did not separate into three distinct fractions on high-speed centrifuging (see below) and did not yield a clear serum after coagulation by deep freezing. The latex was therefore collected at normal temperature in clean aluminium tapping cups. The collection period was limited to 30 min. and the first runnings from the tree were not included in the samples.

Serum was obtained from the latex in two ways. The first consisted in freezing the latex at $-25°$ for a period of 15 days or more (Sekhar & McMullen, 1951). The frozen mass was broken up into small pieces to expose a large surface to the air; after thawing, the rubber separated completely, leaving a clear serum which could be decanted and freeze-dried. The serum so obtained is described here as *F* serum. The method of preparation is essentially the same as that used by Bishop (1927) and by Roe & Ewart (1942). If appreciably less than 15 days' freezing at $-25°$ was allowed, or the frozen latex was not broken up, coagulation was often incomplete and a milky serum resulted.

Alternatively, the freshly tapped latex was treated as rapidly as possible, by centrifuging at 59 000 *g* in an ultracentrifuge at 5°. After 45 min. at speed, the serum had separated as an almost clear 'middle' layer, with a 'heavy fraction' at the bottom and a rubber layer at the top of the centrifuge tube (Cook & Sekhar, 1953). The serum could be run off by making two small punctures in the celluloid centrifuge tube, one near the bottom of the serum layer, the other near the top. This material, described in the present paper as *C* serum, was stored at $-25°$ until a second collection and centrifuging had been made two days later. The sera from the two collections were bulked and freeze-dried together.

When dry, the serum solids were transferred to smaller glass tubes, the moisture absorbed during transfer was removed under vacuum and the tubes sealed off. Samples were stored in this condition, preferably at 0°, until ready for use.

Methods

Elementary analyses. C, H and ash determinations were made by Pregl micro-combustion, and total N by the micro-Kjeldahl method. P was determined colorimetrically with ammonium molybdate, after wet oxidation. The S content of the ash and gaseous products after micro-combustion was determined turbidimetrically, as $BaSO_4$ after complete dissolution of the ash in dil. HCl. All samples were dried to constant weight *in vacuo* at room temp. before analysis.

Protein nitrogen. Three methods were examined for the determination of protein N, namely, precipitation with saturated ammonium sulphate, with 20% (w/v) trichloroacetic acid or with 0·5% tannic acid:

(*a*) A 1% solution of the serum solids was saturated with ammonium sulphate and set aside for 24 hr. at 0°. The precipitate was centrifuged off and freed from sulphate by dialysis in cellophan at 0°. The resulting suspension was then freeze-dried, brought to constant weight at 105° and analysed for N.

(*b*) A 40% (w/v) solution of trichloroacetic acid was added to an equal volume of the serum-solids solution (1%, w/v) and the precipitate centrifuged down. In this case, protein N could be determined conveniently by the difference in N content of the clear supernatant and the original solution. Alternatively, the precipitate was washed by resuspending in dilute trichloroacetic acid and recentrifuging; the product was then dried as in (*a*) and analysed.

(*c*) The same procedure was followed as in (*b*), but a 1% solution of tannic acid was used instead of trichloroacetic acid. Protein N was normally determined by difference. Corrections were made, when necessary, for the N content of the tannic acid (or trichloroacetic acid in (*b*)).

The *C* solids were usually contaminated by small amounts of rubber, present in the original latex as very small particles which were not separated completely at the highest centrifuge speeds attained. On analysis of the precipitated protein directly for N, it was therefore necessary to extract the precipitate with benzene before drying it at 105°.

Paper electrophoresis. For determining the number of electrophoretically distinct proteins in the serum solids, paper electrophoresis on Whatman no. 1 paper was used. The apparatus was a modification of that described by Kunkel & Tiselius (1951), with 0·05 M sodium veronal buffer (pH 8·6) as the solvent medium. To improve resolution of the proteins, the paper was separated from the supporting plates by narrow strips of polythene 0·06 in. thick, parallel to the edges of the paper. After electrophoresis had proceeded under a potential gradient of 4·5 v/cm. for 1000 min., the paper was dyed with 1% bromophenol blue in ethanol, as recommended by Kunkel & Tiselius. Electroendosmotic flow was corrected for by the use of dextran.

After drying, the paper was cut into strips and the amount of dye on each strip determined absorptiometrically after elution with 0·01 M-NaOH. Later, an EEL photoelectric scanner (Evans Electroselenium Ltd., Harlow, Essex), which gave much more rapid results than the dye-elution technique, was used.

The quantitative determination of protein concentration along the paper has not been attempted, in view of the arbitrary assumptions involved in such determinations (Crook, Harris, Hassan & Warren, 1954; Franglen & Martin, 1954).

RESULTS

Analyses of latex-serum solids. An extensive analysis of thirty-three samples, collected over a period of 18 months, is summarized in Table 1.

At concentrations of 5 and 0·5% (w/v) the *F* solutions had pH values of 6·4 ± 0·1 and 6·6 ± 0·1 respectively immediately after dissolution. No change in pH occurred on allowing these solutions to stand in air at 20° for several hours. The pH values of the *C* samples immediately after dissolving were considerably higher and varied between pH 7·3 and 8·1, at 5% concentration. On standing in air at 20°, the pH of these solutions decreased rapidly (Fig. 1), all reaching pH 6·6 ± 0·15 after 3 hr. (curve *b*). An identical rate of fall of pH occurred when the solutions were kept under pure nitrogen. When a freshly prepared solution of the *C* solids was heated at 90° for 5 min. (curve *a*) no subsequent fall in pH occurred after cooling (the heat treatment itself usually produced a small increase in pH of approx. 0·3 unit). The fall of pH in solutions of the *C* solids is probably due in part to production of hydrogen sulphide, which could be detected by lead acetate paper after the solutions had been set aside for half an hour or more in a closed vessel.

Table 1. *Elementary analysis of dry serum solids*

	%								
	C	H	N	S	P	Ash	Protein N*	Non-protein N*	Protein content (see text)*
Average for 27 *F* samples	37·6	6·19	4·01	0·23	1·04	13·5	1·93	2·08	12·8
Standard deviation	0·66	0·10	0·37	0·07	0·25	1·10	—	—	—
Average for 6 *C* samples	39·7	6·43	4·52	0·16	0·74	11·4	2·7	1·82	17·8
Standard deviation	0·38	0·15	0·20	0·02	0·14	1·20	—	—	—

* Determined on samples of average total N content.

With the *F* solids, the three methods of determining protein N, referred to above, gave concordant results. Completeness of precipitation was shown by dialysing the filtrates against water at 0° for 72 hr., after which a negative biuret test was obtained. The N content of the dried precipitate was 15·2% ± 0·2%. For converting protein N into % protein, a conversion factor of 6·58 is therefore recommended for the total proteins.

With solutions of the *C* solids, precipitation of protein by saturated ammonium sulphate was incomplete, the filtrate yielding a further precipitate on heating to 100°. Precipitation with 20% trichloroacetic or 0·5% tannic acid yielded filtrates which gave no further precipitate on heating, and methods (*b*) and (*c*) were therefore used for the protein N determinations. The N contents of the dried products were in good agreement with those found for the precipitated proteins from *F* solids. The same conversion factor of 6·58 can therefore be used to convert protein N into percentage protein. However, the yield of protein from the *C* solids was relatively high (17·8%), corresponding to a protein N content of 2·7%. Determination of the protein N by difference, from analysis of the supernatant, gave the same result (Table 1).

pH *changes in reconstituted serum.* The *F* solids dissolved readily in distilled water to give clear, pale-yellow solutions. *C* solids also dissolved readily, giving almost colourless solutions which were slightly hazy. In both cases the solutions, when freshly prepared, possessed a slight but pleasant odour.

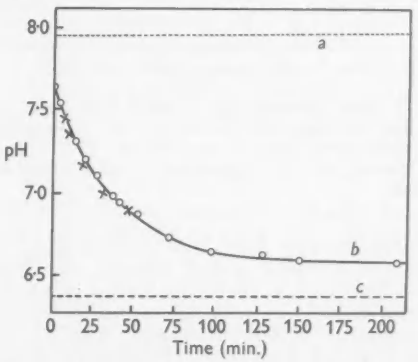

Fig. 1. pH changes in 5% (w/v) solutions of *Hevea* latex-serum solids in distilled water: (*a*) *C* serum heated to 90° and cooled; (*b*) *C* serum in air (○) or N_2 (×); (*c*) *F* serum.

Protein components of reconstituted serum. The number of individual proteins (or proteoses) in the *F* solids, detectable by electrophoresis, was determined by first precipitating the total protein with saturated ammonium sulphate. Excess of sulphate was removed by dialysis at 0° against 0·05 M veronal buffer (pH 8·6). A solution of the total protein in the buffer was then subjected to electrophoresis on paper. A typical electrophoretic diagram is shown in Fig. 2, in which the individual protein fractions have been numbered 1–7 in order of increasing anionic mobility. It is clear that fraction 1 is positively charged at pH 8·6. Fig. 2 shows many resemblances to the schlieren diagrams at pH 8·43 obtained by Roe & Ewart (1942) for the frozen latex serum of Florida and Sumatra latex. The lettering of the individual protein fractions given by these authors appears to be related to ours in the following way (our protein no. 2 was not detected by Roe & Ewart; protein VII described by them has not been detected by us):

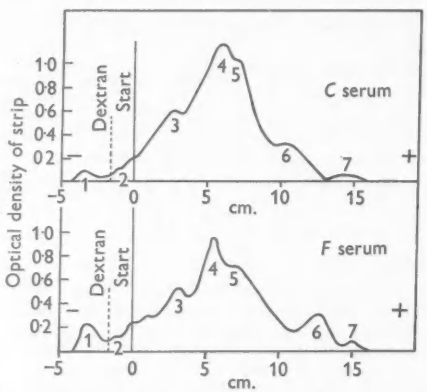

Fig. 2. Paper electrophoresis of *Hevea* latex-serum proteins. Electrophoresis for 1000 min. in 0·05 M sodium veronal buffer (pH 8·6) at 4·5 v/cm. The ordinates represent the optical density of the strip in arbitrary units.

Roe & Ewart	...	I	II	III	IV	V	VI	VII	—
Present work	...	4	6	1	3	5	7	—	2

Since precipitation by trichloroacetic acid or tannic acid results in the irreversible coagulation of some of the protein components, and precipitation by saturated ammonium sulphate is incomplete with the *C* solids, solutions of the latter were prepared for electrophoresis by dialysing, at 0°, a concentrated solution of the solids against 0·05 M veronal buffer (pH 8·6) for 24 hr. A typical electrophoretic diagram is included in Fig. 2. Protein no. 7 has not been detected in all samples tested.

Effect of ammonia on the proteins of F serum. Roe & Ewart (1942) found that when the serum from frozen latex was treated with ammonia, the number of protein components detectable by electrophoresis decreased until, after several months, only two components of the original seven could be detected. Although it is not entirely

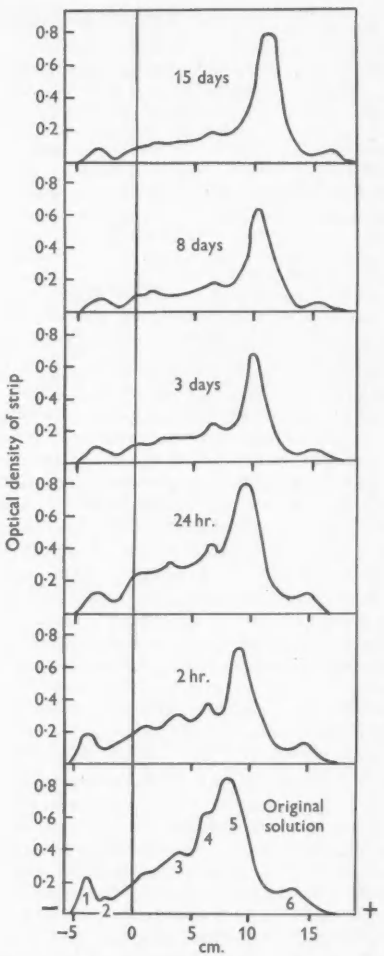

Fig. 3. Effect of ammonia (1·2%, w/v) on the electrophoresis diagram of *F* serum. Initial position of the proteins is indicated by the vertical line. The times refer to the period of contact with ammonia before electrophoresis. Electrophoresis carried out in 0·05 M sodium veronal buffer (pH 8·6) for 1000 min. at 4·5 v/cm.

clear which these two components were, the one present in the larger concentration appeared to be either protein 4 or 5, in our nomenclature. It was noticed that the ammonia also produced a gradual increase in the anionic mobility of this constituent.

In the present work, samples of the same sample of *F* solids were stored in 1·2 % (w/v) aqueous NH_3 at 18° for different times. The solutions were then neutralized with dilute H_2SO_4 and the total protein precipitated by saturation with ammonium sulphate. After centrifuging, the precipitates were mixed to pastes with water and dialysed against 0·05 M veronal buffer, and the resulting solutions analysed by paper electrophoresis. The results (Fig. 3) show:

(*a*) The concentration of one of the main protein components (no. 4) of the original solution decreases rapidly and is barely discernible after 15 days' ammoniation. The concentration of the other main component (no. 5) appears to be largely unaffected by the ammonia.

(*b*) Components 2 and 3 are no longer detectable after 15 days' ammoniation.

(*c*) The concentrations of components 1 and 6 diminish slowly.

(*d*) The anionic mobilities of components 5 and 6 increase, and the cationic mobility of component 1 decreases slightly, as the time of contact with ammonia increases. The mobility of component 4 does not change appreciably with time.

DISCUSSION

It is apparent from Table 1 that the percentage of oxygen in fresh latex-serum solids must be high (about 40 %), as would be expected from the fact that the polyhydric alcohol quebrachitol is known to be a major constituent of the serum (Tunnicliffe, 1954). At least part of the difference between the carbon and hydrogen contents of the *F* and *C* material was due to the *C* material still containing small amounts of rubber hydrocarbon. The ratio of protein nitrogen to total nitrogen is approximately 50 % in the *F* and 60 % in the *C* solids. The difference in the actual protein contents indicates that nearly 30 % of the serum protein in fresh latex is lost by coagulation during long freezing of the latex. The possibility of some hydrolysis of protein during this process cannot be ruled out, in view of the slightly higher non-protein nitrogen content of the *F* solids. However, the fact that the phosphorus content is also appreciably higher in the *F* material suggests that it is phospholipid, initially associated with the rubber particles, which is partially hydrolysed during the freezing process with passage of phosphorus and base nitrogen into the serum phase. The ready hydrolysis of latex phospholipids has been demonstrated by previous workers (Tunnicliffe, 1954; Smith, 1953).

The decrease in the pH value of solutions of the *C* solids in distilled water is attributed to the presence of an acid-producing enzyme or bacteria. With the *F* material, this enzymic or bacterial action has already proceeded sufficiently far, apparently, to give a constant pH of approximately 6·5. Roe & Ewart (1942) also observed that the pH of unammoniated serum prepared from frozen latex was 6·4 and that the serum could be kept at −20° for several months without change.

The electrophoretic diagrams for the *F* and *C* solids (Fig. 2) are very similar, indicating that the difference between the solids is mainly one of protein concentration. However, a difference in the nature of one or more proteins is indicated by the incomplete precipitation of protein from the *C* solids by saturation with ammonium sulphate and the appearance of a further precipitate on heating to 100°. From Fig. 2, proteins nos. 4 and 5 appear to be the main components in both *F* and *C* solids.

Our results showing the effect of ammonia on the proteins of *F* serum are consistent with those of Roe & Ewart (1942), if allowance is made for the different storage periods. The two proteins observed by these authors after prolonged ammoniation were presumably components 1 and 5. The predominating importance, as regards concentration, of protein no. 5 in the ammoniated serum is apparent.

SUMMARY

1. The aqueous serum phase of unpreserved rubber latex (*Hevea brasiliensis*) has been separated in two ways: (*a*) by high-speed centrifuging, and (*b*) by prolonged freezing at −25°. The protein contents of the serum solids are approximately 18 and 13 % respectively; the difference is attributed to coagulation of protein during prolonged freezing of the latex.

2. There is evidence that organic phosphorus compounds (probably phospholipids) are partially hydrolysed during the freezing process, and that enzymic or bacterial reactions occur, leading to a fall in pH of the serum.

3. In both types of serum, seven electrophoretically distinct protein components have been detected, one of which is positively charged at pH 8·6.

4. After treatment with dilute ammonia solution for 15 days, only two of the original protein components are readily resolvable electrophoretically. One of these (no. 5) is a major component of the unammoniated serum.

This work forms part of a programme of research undertaken by the Boards of the Rubber Research Institute of

Malaya and the British Rubber Producers' Research Association. The authors wish to thank Dr E. G. Cockbain for his interest in this work and for his help in the preparation of this paper. Our thanks are also due to Mr Ho Kam Leng for assistance in some of the experimental work, to Mr G. W. Drake and Dr A. I. McMullen for many useful discussions, and to Dr W. T. Chambers and Mr R. J. Thompson for most of the elementary analyses.

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The Proteins of *Hevea brasiliensis* Latex

2. ISOLATION OF THE α-GLOBULIN OF FRESH LATEX SERUM

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In part 1 of this series (Archer & Sekhar, 1955) some properties of the aqueous serum obtained by freezing or centrifuging the fresh latex of *Hevea brasiliensis* were reported and the number of individual protein fractions identifiable by paper electrophoresis was determined. The present investigation is concerned with the isolation and properties of one of the two main constituents, namely component 5, which is shown to be the chief globulin component of fresh latex serum. To avoid confusion between the lettering and numbering systems of different workers, it is proposed that protein 5 be referred to as the α-globulin component of latex serum. It appears to correspond to component V in the nomenclature of Roe & Ewart (1942) and was almost certainly the main constituent of 'protein A' described by Bishop (1927), Kemp & Straitiff (1940) and Bondy & Freundlich (1938).

EXPERIMENTAL

Materials

Freeze-dried serum solids prepared by the Rubber Research Institute of Malaya were used throughout. The aqueous serum phase of fresh unammoniated latex was obtained by prolonged freezing or by centrifuging the latex, the serum being then freeze-dried, in the way already described (Archer & Sekhar, 1955). As before, *F* solids and *C* solids refer to the serum solids obtained from fresh serum prepared by the freezing and centrifuging techniques respectively.

Methods

Elementary analyses. N was determined by the micro-Kjeldahl method, and C, H and ash by Pregl microcombustion. The S content of the ash and gaseous combustion products was measured turbidimetrically as $BaSO_4$, after complete dissolution of the ash in dil. HCl. P was determined colorimetrically with ammonium molybdate, after wet oxidation. All samples were dried to constant weight at room temp. *in vacuo*, before analysis.

Electrophoresis. Paper-electrophoresis measurements were carried out as described in Part 1 (Archer & Sekhar, 1955), 0·05 M sodium veronal buffer (pH 8·6) being used. Measurements in the Tiselius apparatus were made at 0° and current 5 mA. Solutions for electrophoresis were prepared in veronal buffer (pH 8·6, ionic strength 0·1), the solutions being dialysed against a large volume of the buffer for 24 hr. at 0° before use.

Fractionation of serum proteins. Two methods were examined:

(*a*) Following Kemp & Straitiff (1940), solutions of the *F* and *C* serum solids, in distilled water, were partially precipitated with different concentrations of ammonium sulphate at 0°. After 16 hr., the precipitates were centrifuged down and dialysed for 24 hr. against 0·05 M veronal buffer (pH 8·6) at 0°. The resulting solutions were analysed by paper electrophoresis.

(*b*) Total protein was first precipitated from a solution of *F* solids in water by saturation with ammonium sulphate, and the precipitate dialysed against 0·01 M trisodium citrate at 0° until free from sulphate. The resulting protein solution was clarified centrifugally and its N content determined. Portions of the solution were then diluted with sodium citrate buffers so as to cover the pH range 4·0–5·2

at ionic strengths varying from 0·02 to 0·25, or the pH range 2·1–3·6 at ionic strength 1·0 (due allowance was made for the trisodium citrate in the protein solution after dialysis). After centrifuging down the precipitates, the clear supernatants were analysed for N and their pH values checked by means of a glass electrode. All processing was carried out at 0°. The precipitates were dissolved in 0·1 M veronal buffer (pH 8·6) and examined for homogeneity by paper electrophoresis.

Isolation of purified α-globulin. The procedure finally adopted for isolating the α-globulin of the latex-serum solids was based on method (*b*) above. Approx. 40 g. of *F* solids were dissolved in an equal weight of water, to give a practically clear solution. Saturated aqueous ammonium sulphate (A.R., 600 ml.) was then added and the mixture maintained at 0° for 1 hr. The precipitated protein was centrifuged off and washed by resuspending in fresh saturated ammonium sulphate. The recentrifuged protein was stirred to a cream with a little distilled water, and dialysed in cellophan bags against 0·01 M trisodium citrate at 0° for 48 hr., with several changes of the latter solution, to remove most of the contaminating ammonium sulphate. The resulting solution was centrifugally clarified and diluted with 0·01 M trisodium citrate to give protein N approx. 1·5 mg./ml. The solution was then mixed with 2 vol. of a mixture of equal volumes of 0·0032 M trisodium citrate and 0·054 M sodium dihydrogen citrate to give a final pH of 4·5. All solutions were cooled to 0° before use.

The precipitated α-globulin was centrifuged off immediately, stirred to a smooth cream with a little water and redissolved by the gradual addition of 0·1 N-NaOH with constant stirring. The pH of the mixture was not allowed to rise above 7·0. Any protein remaining undissolved at this pH was rejected. Provided that the time during which the protein–buffer mixture was kept at pH 4·5 did not exceed 30 min., and the temperature was kept at approx. 0°, the amount of residue insoluble at pH 7·0 was very small. Washing the precipitated globulin with fresh pH 4·5 buffer tended to decrease the solubility of the product.

The solution of α-globulin was reprecipitated three times at pH 4·5 under the same conditions as those described above. The proportions of the two citrate solutions added were adjusted to allow for the amount of caustic soda used in dissolving the protein. The final solution was then freeze-dried or preferably used at once, since the solubility of a dry sample of the protein tended to fall on storage, even at 0°.

The serum solids obtained from fresh latex by centrifuging (*C* solids) usually still contained approx. 0·5% of rubber of very small particle size, which redispersed when the solids were dissolved in water. To prevent contamination of the α-globulin with this residual rubber, the *C* solids were first dialysed in a cellophan bag against 0·01 M trisodium citrate solution at 0° for 72 hr. The resulting solution contained the rubber in a slightly flocculated state; most of it could be removed by centrifuging at 20 000 *g*. The last traces of rubber were removed by the addition of ammonium sulphate until the concentration was between 20 and 30% saturated (a small amount of protein was also precipitated). The protein solution was then clarified by centrifuging and the α-globulin isolated as described above, starting with the precipitation by saturated ammonium sulphate.

Results

Fractionation with ammonium sulphate. Fig. 1 shows the paper-electrophoresis diagrams for fractions i–iv of *F* serum solids obtained by successive precipitation with 25, 50, 70 and 100% saturated ammonium sulphate respectively. Fraction i appears to consist mainly of proteins 5 (α-globulin) and 3, in the nomenclature of Archer & Sekhar (1955), but the amount of this fraction is small. Fractions ii and iii contain most of the α-globulin, but are very heterogeneous. Fraction iv consists mainly of two components, one of which is protein 4, and contains little or no α-globulin. Similar results were obtained by fractionation of the *C* solids.

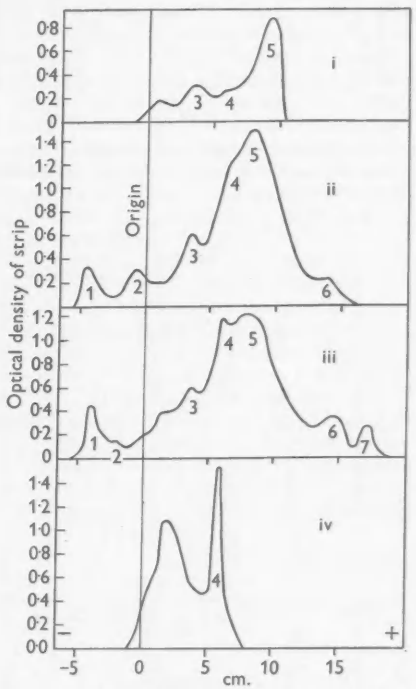

Fig. 1. Paper electrophoresis diagrams of protein fractions from *F* solids by precipitation with ammonium sulphate. Electrophoresis was carried out for 1000 min. in 0·05 M sodium veronal buffer (pH 8·6); potential gradient 4·5 v/cm. (i), 0–25%; (ii), 25–50%; (iii), 50–70%; (iv), 70–100% saturated ammonium sulphate. The arabic numerals refer to the components of Archer & Sekhar (1955).

Solubility of serum proteins in citrate buffer. The over-all solubility of *F* serum proteins in citrate buffer at 0°, as a function of pH and ionic strength (*I*), is shown in Fig. 2. The pH of minimum solubility is well-defined at $I = 0{\cdot}02$ or 0·04, but less so at higher ionic strengths. Above

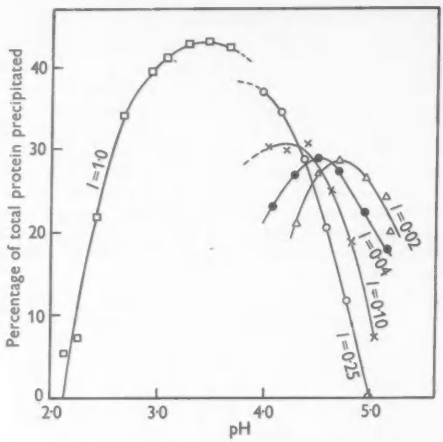

Fig. 2. The effect of pH and ionic strength on the percentage of *F* serum proteins precipitated from sodium citrate buffer at 0°.

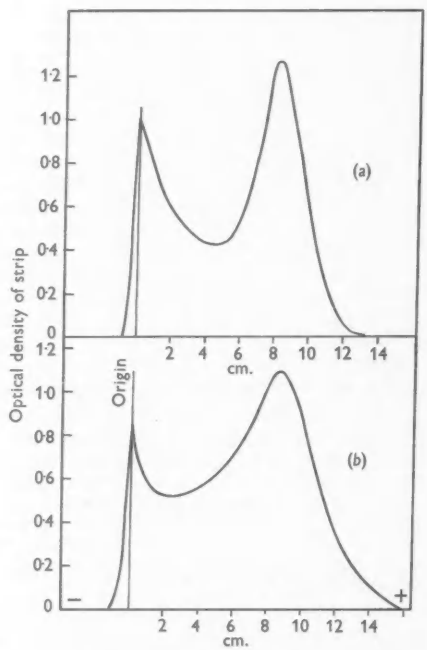

Fig. 3. Paper-electrophoresis diagrams of α-globulin isolated from (*a*) *F* solids and (*b*) *C* solids. Electrophoresis was carried out for 1000 min. in 0·05 M sodium veronal buffer (pH 8·6); potential gradient 4·5 v/cm.

approximately pH 4·5, the solubility of the total protein increases with increasing ionic strength, while at lower pH values the reverse is true. The pH of minimum solubility falls from 4·7 at $I=0{\cdot}02$, or 4·5 at $I=0{\cdot}04$, to about 3·4 at $I=1{\cdot}0$. Paper-electrophoretic measurements indicated that an increase in ionic strength gave precipitates of increasing heterogeneity. These results apply to solutions in which the initial protein concentration was low (approx. 0·2%). Further experiments at pH 4·5 showed that a gradual increase in the heterogeneity of the precipitates occurred if the initial protein-N content of the solutions exceeded 0·5 mg./ml. Use was made of these results in choosing conditions for the isolation of the α-globulin, as described in a previous section.

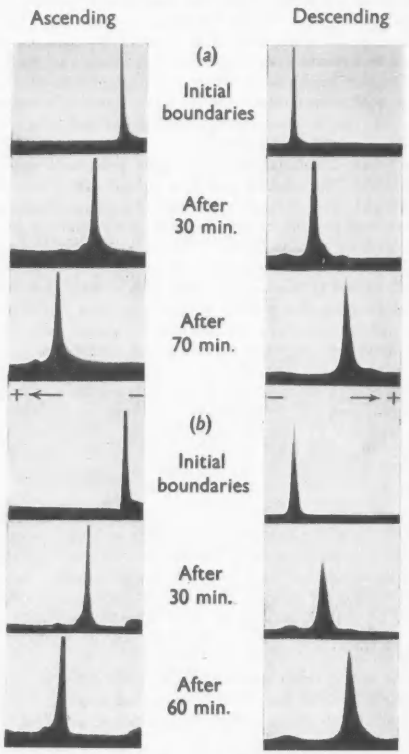

Fig. 4. Schlieren diagrams of α-globulin from (*a*) *F* solids and (*b*) *C* solids. Tiselius electrophoresis was carried out at 0° and 5 mA, in sodium veronal buffer (pH 8·6, *I* 0·1).

α-Globulin of latex serum. Typical paper-electrophoresis diagrams for the freshly prepared α-globulin of *F* and *C* serum solids are given in Fig. 3. Pronounced 'tailing' of the α-globulin spot occurs, when veronal buffer at pH 8·6 is used, owing to the ease with which the protein is adsorbed on filter paper. The globulin of the *F* solids is indistinguishable from that of *C* solids, and the adsorption prevents

ready detection of heterogeneity due to slower-moving components. However, using the Tiselius apparatus, the schlieren diagrams (Fig. 4) showed the presence of two minor components in both preparations, amounting to approx. 10% of the total. The faster-moving impurity is probably protein 6, detected previously by Archer & Sekhar (1955).

Elementary analysis. Analytical data for α-globulin (from *F* solids), after exhaustive dialysis against distilled water, are given in Table 1. Also included in the Table are analyses by other workers of the 'protein A', the main component of which is believed to be α-globulin.

Table 1. *Elementary analyses of α-globulin and 'protein A'*

		'Protein A'	
Element	α-Globulin (%)	Bishop (1927) (%)	Kemp & Straitiff (1940) (%)
N	15·7±0·1	14·0–15·1	12·7
C	52·1	—	50·7
H	6·7	—	7·3
S	0·06	—	1·32
P	<0·01	—	0·01
Ash	0·4	0·2–0·5	2·26

Qualitative analysis of the ash showed that the chief metal present was Na (probably left from the sodium citrate buffer used in isolating the protein) with traces of Mg, K and Ca.

Colour and precipitation reactions. Positive reactions to the following tests were obtained: biuret, ninhydrin, xanthoproteic, Millon, Sakaguchi and Adamkiewicz. Reactions to the Molisch and Benedict tests were negative, indicating the absence of carbohydrate. These results are in agreement with the findings of Bishop (1927), Bondy & Freundlich (1938) and Kemp & Straitiff (1940), except that the last authors reported a negative Millon test.

Solubility and isoelectric range of α-globulin. When freshly prepared, α-globulin is readily soluble in neutral or alkaline salt solutions and in acid solutions of pH less than about 3. It is insoluble in distilled water. Determinations of solubility were made over the pH range 4·1–5·9, in sodium acetate buffers of ionic strength 0·04, with a freshly prepared solution of the protein which had been exhaustively dialysed against 0·01 M sodium acetate solution. All measurements were carried out at 0°. Between pH 4·3 and 5·4, the solubility of the protein was extremely small (approx. 0·03 g./l.); very similar results were obtained at ionic strength 0·1, except that the solubility was slightly greater. On each side of these pH limits, the solubility increased very rapidly. From these solubility characteristics, the protein can be classified as a globulin with an isoelectric point in the region of 4·8.

The effect of temperature on the solubility of the protein was examined over the pH range 5·5–8·7, the ionic strength being varied by means of sodium acetate. At $I = 0{\cdot}07$, the coagulation produced by heating at 70° for 2 hr. was almost complete (>94%) at pH values between 5·5 and 7·0. Under the same conditions at pH 8·7, 80% of the protein was coagulated. As the ionic strength was decreased, the percentage of protein coagulating decreased, becoming negligibly small (at pH 7) at ionic strengths less than 0·01.

DISCUSSION

Kemp & Straitiff (1940) isolated 'protein A' by half-saturation of fresh latex serum with ammonium sulphate. From Fig. 1, it is clear that this procedure yields a very heterogeneous fraction, containing all but one of the seven protein components detected by Archer & Sekhar (1955) in fresh serum solids. The electrophoretic diagrams for fractions precipitated at other concentrations of ammonium sulphate show that the method is not suitable for purifying the α-globulin component. The difference in composition between α-globulin and Kemp & Straitiff's fraction (Table 1) is particularly marked in respect of nitrogen, sulphur and ash contents, although the higher values for sulphur obtained by Kemp & Straitiff may be due in part to their preparation not being completely freed from ammonium sulphate.

The 'protein A' isolated by Bishop (1927) from acidified latex serum was reported to contain phosphorus, whereas α-globulin has a negligible phosphorus content after thorough dialysis of the protein. Bishop's data for the ash content of his 'protein A' is in good agreement with that of α-globulin. However, it is probable that the material obtained by Bishop was at least partly denatured, since its solubility was reported to be small except at very high pH values. When freshly prepared, α-globulin is readily soluble in salt solutions at pH 7, and in latex serum reconstituted from *F* or *C* serum solids.

The value of 15·7% for the nitrogen content of α-globulin, the negligible phosphorus content and the negative reaction for carbohydrate indicate that it is not a conjugated protein.

SUMMARY

1. One of the two major protein components of fresh *Hevea*-latex serum has been isolated by an isoelectric precipitation method and the optimum conditions for the precipitation have been determined.

2. Attempts to isolate this component by fractional precipitation of the total serum proteins with ammonium sulphate gave markedly heterogeneous products.

3. A preliminary examination has been made of the purified protein. Analytical and solubility data indicate that it is a globulin lacking non-protein prosthetic groups.

This work forms part of a programme of research undertaken by the Board of the British Rubber Producers' Research Association. Our thanks are due to the Rubber Research Institute of Malaya for the supplies of latex serum solids used in the investigation, to Dr A. D. Patrick for carrying out the electrophoretic analyses shown in Fig.4, and to Professor M. Stacey for permission to use the Tiselius apparatus at Birmingham University.

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Studies in Detoxication

66. THE METABOLISM OF HALOGENOBENZENES. 1:2:3-, 1:2:4- AND 1:3:5-TRICHLOROBENZENES*

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The trichlorobenzenes are decomposition products of the insecticide benzene hexachloride (gammexane). On treatment with alkali, the α-, β-, γ- and δ-isomers of benzene hexachloride are dehydrochlorinated to yield a mixture of the three isomeric trichlorobenzenes in which the 1:2:4-isomer predominates and amounts to about 80% of the total (cf. Hughes, Ingold & Pasternak, 1953). Benzene hexachloride also decomposes under field conditions, and it has been shown that the deleterious effects of crude benzene hexachloride on wheat seedlings and coniferous seedlings may be due to trichlorobenzenes, the 1:2:4-isomer being the most active towards wheat and the 1:3:5-isomer towards coniferous seedlings (Hocking, 1950; Simkover & Shenefelt, 1952). Benzene hexachloride is also decomposed in animals and insects, but nothing is known about the nature of the metabolites. However, it is known that the different isomers of benzene hexachloride are metabolized at different rates in mice and in flies (Asperen, 1954; Oppenoorth, 1954, 1955). When the common isomers of benzene hexachloride are fed to rats over a long period, the foreign material which accumulates in the tissues, particularly the fat, does not contain 1:2:4-trichlorobenzene, but consists of unchanged benzene hexachloride (Davidow & Frawley, 1951).

The present work was undertaken with the view in mind that benzene hexachloride may be converted into trichlorobenzenes *in vivo* and that these may be further metabolized to phenolic substances. A knowledge of the metabolism of the trichlorobenzenes could thus be useful in elucidating that of benzene hexachloride. The trichlorobenzenes themselves have been used as pesticides and they are also employed in industry for various purposes such as the manufacture of electrical insulating material. From a toxicological point of view, the trichlorobenzenes are less toxic to small laboratory animals than chlorobenzene and *o*-dichlorobenzene (Cameron *et al.* 1937), and this may be correlated with the extent of mercapturic acid formation, which diminishes as the number of chlorine atoms in these compounds increases.

The metabolism of the isomeric dichlorobenzenes in rabbits has already been described (Azouz, Parke & Williams, 1955; Parke & Williams, 1955).

* Part 65: Smith & Williams (1955).

EXPERIMENTAL

Materials and reference compounds

1:2:3-Trichlorobenzene, m.p. 53°, was purchased (L. Light and Co., Colnbrook, Bucks.); 1:2:4-trichlorobenzene, m.p. 17°, and 1:3:5-trichlorobenzene, m.p. 61°, were prepared according to Holleman (1918).

The following compounds were prepared by standard methods (m.p.'s are corrected; references to the known compounds are given by Huntress (1948)).

Compounds related to 1:2:3-*trichlorobenzene.* 2:3:4-Trichlorophenol, m.p. 81°, and benzoate, m.p. 142°; and 3:4:5-trichlorophenol, m.p. 98°, and benzoate, m.p. 119°, were prepared.

3:4:5-Trichlorocatechol was synthesized as follows. 2:3:4-Trichlorophenol was converted into 3:4:5-*trichlorosalicylaldehyde* as described by Duff (1941) for the preparation of *o*-hydroxyaldehydes. The aldehyde (yield 20%), m.p. 87°, formed yellow needles from aqueous ethanol. (Found: C, 37·4; H, 1·6; Cl, 47·0. $C_7H_3O_2Cl_3$ requires C, 37·3; H, 1·3; Cl, 47·2%.) The *semicarbazone*, m.p. 236°, formed pale-yellow needles from aqueous ethanol. (Found: C, 34·1; H, 2·4; Cl, 37·7. $C_8H_6O_2N_3Cl_3$ requires C, 34·0; H, 2·1; Cl, 37·7%.) The aldehyde in N-NaOH was treated with H_2O_2, as described by Dakin (1909) for the oxidation of *o*-hydroxyaldehydes, to yield 3:4:5-trichlorocatechol (yield

Table 1. *Synthetic hexachlorodiphenyl disulphides*

Trichloroaniline* used	Hexachlorodiphenyl disulphide isolated: Isomer	M.p.	Analysis (found): C	H	Cl
2:3:4-	2:3:4:2′:3′:4′-	185°	34·0	0·9	50·4
3:4:5-	3:4:5:3′:4′:5′-	152°	34·1	1·1	50·2
2:3:5-	2:3:5:2′:3′:5′-	168°	34·2	1·0	50·0
2:4:5-	2:4:5:2′:4′:5′-	136–137°	34·0	1·1	50·3
2:4:6-	2:4:6:2′:4′:6′-†	173°	33·8	0·9	49·9
	($C_{12}H_4Cl_6S_2$ requires		33·9	0·95	50·1)

* See text.

† This compound has previously been described by Baddeley & Bennett (1933), who record m.p. 165°.

80%), m.p. 105° [Huntress (1948) gives m.p. 106–109° and the compound is described as the hemihydrate], which was chromatographically pure. Its *dibenzoate*, m.p. 114–115°, formed colourless needles from aqueous ethanol. (Found: C, 57·1; H, 2·65; Cl, 25·3. $C_{20}H_{11}O_4Cl_3$ requires C, 57·0; H, 2·6; Cl, 25·2%.)

Compounds related to 1:2:4-*trichlorobenzene.* 2:3:5-Trichlorophenol, m.p. 62°, and its benzoate, m.p. 103°; 2:3:6-trichlorophenol, m.p. 58°, and its benzoate, m.p. 90°; 2:4:5-trichlorophenol, m.p. 68°, and its benzoate, m.p. 92°; and 2:3:5-trichloroquinol, m.p. 136°, were prepared.

3:4:6-Trichlorocatechol was synthesized. 2:4:5-Trichlorophenol was converted into 3:4:6-*trichlorosalicylaldehyde* as described above. The aldehyde formed yellow needles, m.p. 116°, from aqueous ethanol; yield 20%. (Found: C, 37·3; H, 1·5; Cl, 46·8%.) The *semicarbazone* was obtained as yellow needles, m.p. 244°, from aqueous ethanol. (Found C, 33·8; H, 1·9; Cl, 37·4%.) This aldehyde was oxidized to 3:4:6-*trichlorocatechol* (yield 80%) as above. It formed white needles of the hemihydrate, m.p. 92°, from water. (Found: C, 32·7; H, 2·1; Cl, 47·7. $C_6H_3O_2Cl_3,0{\cdot}5H_2O$ requires C, 32·3; H, 1·8; Cl, 47·8%.)

Compounds related to 1:3:5-*trichlorobenzene.* 2:4:6-Trichlorophenol, m.p. 67°, and its benzoate, m.p. 74°; 2:4:6-trichlororesorcinol, m.p. 83°; and 2:4:6-trichlorophloroglucinol, m.p. 134°, were prepared.

Synthesis of hexachlorodiphenyl disulphides. A cold mixture of sulphuric acid (8 ml.) and water (8 ml.) was added to a solution of the appropriate trichloroaniline (2 g.) in glacial acetic acid (4 ml.) and the whole cooled to 0°. The aniline was diazotized by gradual addition of solid sodium nitrite (1·4 g.). Ethyl potassium xanthate (2 g.) was slowly added in small quantities. The mixture turned yellow and a solid separated. On keeping in the ice bath, which was allowed to reach room temperature gradually, the solid decomposed with evolution of nitrogen. The oil which formed was separated, dissolved in ether and freed from phenol by washing with 2N-NaOH. The ethereal solution was then washed with 2N-H_2SO_4 and finally with water. The ether was evaporated and the oily residue dissolved in ethanol (20 ml.) and refluxed with solid KOH (0·7 g.) for 10–20 min. The ethanol was distilled off and the residue acidified with 2N-H_2SO_4, to liberate the trichlorothiophenol, which was steam-distilled and obtained as a waxy solid from the distillate by filtration. The thiophenol was dissolved in ethanol (10–15 ml.) and titrated with a 5% solution of iodine in ethanol. The *hexachlorodiphenyl disulphides* (0·4 g.) separated as white crystalline solids which were purified by recrystallization from aqueous ethanol or ethanol–ethyl acetate mixtures. Five of these disulphides were synthesized (see Table 1) and they crystallized as colourless needles, almost insoluble in water, sparingly soluble in cold ethanol but more soluble in hot ethanol.

Methods

Animals. Chinchilla rabbits, kept on a diet of 60 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water per day, were used throughout this work. The trichlorobenzenes were administered by stomach tube as 25% (w/v) solutions in arachis oil. Urine was collected daily.

Analytical methods. The urine was analysed daily for glucuronic acid by the modified naphthoresorcinol method of Paul (1951), ethereal sulphate by the turbidimetric method of Sperber (1948), and mercapturic acid by the iodine-titration method of Stekol (1936).

Determination of trichlorophenols. The total trichlorophenol was estimated spectrophotometrically by determination of the difference between the acid and alkaline spectra of steam distillates of the acid-hydrolysed urines as described by Azouz *et al.* (1955) for dichlorophenols. Chromatography of the phenolic metabolites of 1:2:3-trichlorobenzene showed that, compared with 2:3:4-trichlorophenol, the 3:4:5-isomer was a relatively minor constituent, and therefore the total phenol was estimated at 246 mμ. and calculated as 2:3:4-trichlorophenol. The spectra in acid and alkali of 2:3:4- and 3:4:5-trichlorophenols are shown in Fig. 1 (for 2:3:4-trichlorophenol ε at 246 mμ. is 7100 in 0·1N-NaOH and 1100 in 0·1N-HCl and for 3:4:5-trichlorophenol it is 10 700 in 0·1N-NaOH and 1050 in 0·1N-HCl). Recoveries of 2:3:4- and 3:4:5-trichlorophenols from normal rabbit urine at concentrations of 25 mg./100 ml. were 93±5% and 97±5% respectively.

Chromatography of the phenolic metabolites of 1:2:4-trichlorobenzene showed that only 2:3:5- and 2:4:5-trichlorophenol were present, in approximately equal amounts (see below), and the total phenol was estimated at 244 mμ. and calculated as 2:4:5-trichlorophenol. The spectra in acid and alkali of 2:3:5- and 2:4:5-trichlorophenols are shown in Fig. 2 (for 2:3:5-trichlorophenol ε at 244 mμ. is 7250 in 0·1N-NaOH and 1100 in 0·1N-HCl and for 2:4:5-trichlorophenol it is 8900 in 0·1N-NaOH and 1100 in 0·1N-HCl). Recoveries of 2:3:5- and 2:4:5-trichlorophenols from normal rabbit urine at concentrations of 25 mg./100 ml. were 95±5% and 100±5% respectively.

2:4:6-Trichlorophenol, the only monophenol obtained from 1:3:5-trichlorobenzene, was estimated at 244 mμ. The spectra of this phenol in acid and alkali are shown in Fig. 3 (ε at 244 mμ. is 8950 in 0·1 N-NaOH and 1050 in 0·1 N-HCl). Recovery of 2:4:6-trichlorophenol from normal rabbit urine at a concentration of 25 mg./100 ml. was 95±5%. The recoveries of the trichlorophenols (25 mg./100 ml.) from normal rabbit urine were completely unaffected by the addition of 25–75 mg. of glucurone/100 ml. of urine.

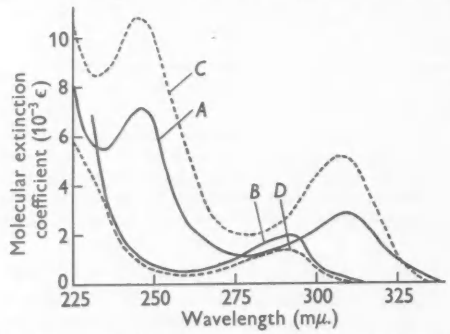

Fig. 1. Spectra of 2:3:4- and 3:4:5-trichlorophenols. *A*, 2:3:4-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 246, 309 mμ.; $\epsilon_{max.}$ 7100, 2800) and, *B*, in 0·1 N-HCl ($\lambda_{max.}$ 293 mμ.; $\epsilon_{max.}$ 1950); *C*, 3:4:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 243, 307 mμ.; $\epsilon_{max.}$ 10 800, 5050) and, *D*, in 0·1 N-HCl ($\lambda_{max.}$ 290 mμ.; $\epsilon_{max.}$ 1300).

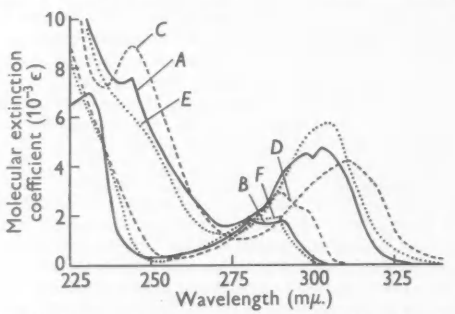

Fig. 2. Spectra of 2:3:5-, 2:4:5- and 2:3:6-trichlorophenols. *A*, 2:3:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 298, 302 mμ.; $\epsilon_{max.}$ 7250, 4450, 4700) and, *B*, in 0·1 N-HCl ($\lambda_{max.}$ 280, 290 mμ.; $\epsilon_{max.}$ 1900, 1900); *C*, 2:4:5-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 310 mμ.; $\epsilon_{max.}$ 8900, 4200) and, *D*, in 0·1 N-HCl ($\lambda_{max.}$ 290 mμ.; $\epsilon_{max.}$ 2900); *E*, 2:3:6-trichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 304 mμ.; $\epsilon_{max.}$ 5650) and, *F*, in 0·1 N-HCl ($\lambda_{max.}$ 280, 289 mμ.; $\epsilon_{max.}$ 1900, 1950).

Chromatography

1:2:3-*Trichlorobenzene metabolites.* The possible phenolic metabolites of 1:2:3-trichlorobenzene are 2:3:4- and 3:4:5-trichlorophenol, and 3:4:5-trichlorocatechol. The monophenols could be separated from the catechol by steam distillation. Two solvent systems were used: A, benzene–acetic acid–water (1:1:2, v/v) and B, the *n*-butanol–ethanol–ammonia–ammonium carbonate buffer system of Fewster & Hall (1951). The two monophenols were not completely separated in these solvents, but could readily be distinguished by their colour reactions with diazotized *p*-nitraniline and sulphanilic acid (see Table 2). Convenient amounts of phenols for chromatography were of the order of 50 μg.

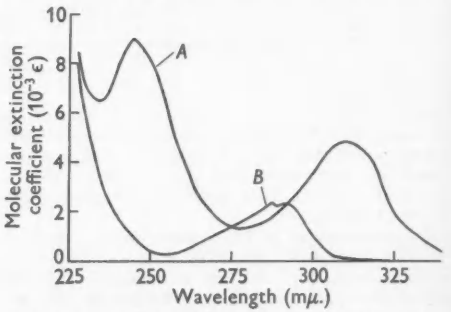

Fig. 3. Spectra of 2:4:6-trichlorophenol. *A*, in 0·1 N-NaOH ($\lambda_{max.}$ 244, 311 mμ.; $\epsilon_{max.}$ 8950, 4900) and *B*, in 0·1 N-HCl ($\lambda_{max.}$ 287, 292 mμ.; $\epsilon_{max.}$ 2350, 2300).

1:2:4-*Trichlorobenzene metabolites.* The possible phenolic metabolites are: 2:3:5-, 2:4:5- and 2:3:6-trichlorophenol; 3:4:6-trichlorocatechol and 2:3:5-trichloroquinol. The monophenols could be separated from the polyphenols by steam distillation and then on paper in solvent system B (Table 2). The 2:3:6-trichlorophenol could be separated from the other two monophenols, which, though not themselves separable in either solvent system, could be distinguished by colour reactions (see Table 2). The polyphenols were readily separable in solvent system A.

1:3:5-*Trichlorobenzene metabolites.* The possible phenolic metabolites are 2:4:6-trichlorophenol, 2:4:6-trichlororesorcinol and trichlorophloroglucinol. The monophenol was readily separated from the polyphenols by steam distillation, and the polyphenols were readily separable from each other in solvent system A (see Table 2).

Isolation and detection of metabolites

From 1:2:3-*trichlorobenzene*

Monophenols. A total of 4·5 g. of 1:2:3-trichlorobenzene was fed to three rabbits and their urine collected for 5 days. The neutral, non-reducing urine gave an intense naphthoresorcinol reaction and a blue colour with 2:6-dichloroquinonechloroimide. The urine was brought to pH 3 with HCl and extracted with ether for 5 hr. The phenols in the extract were transferred to N-NaOH (2 × 25 ml.), which was then acidified and steam-distilled. The colourless oil (50 mg.) in the distillate crystallized, m.p. 80°, and was benzoylated. The crystalline benzoate was identified after recrystallization as 2:3:4-trichlorophenyl benzoate, m.p. and mixed m.p. 142°. The residual urine was now made N with respect to HCl and heated on a water bath for 0·5 hr.

Table 2. *The R_F values and colour reactions on paper of trichlorophenols*

Figures in parentheses are pH values.

Phenol	R_F values in* A	R_F values in* B	Colour reactions: Gibbs reagent†	Diazotized‡ *p*-nitraniline	Diazotized sulphanilic acid§	Brentamine Fast Red B salt‖	$FeCl_3$¶
2:3:4-Trichlorophenol	0·92	0·83	Blue green (8)	Violet	Yellow	Violet	—
3:4:5-Trichlorophenol	0·88	0·89	Pale blue (10)	Orange	Orange	Orange	—
3:4:5-Trichlorocatechol	0·55	0·76	Green (8)	Buff	—	—	Green (7) Violet (9)
2:3:5-Trichlorophenol	0·90	0·84	Blue (8)	Orange	Orange	Orange	—
2:3:6-Trichlorophenol	0·92	0·73	Blue (8)	Magenta	Yellow	Magenta	—
2:4:5-Trichlorophenol	0·92	0·83	Blue (8)	Violet	Orange	Violet	—
3:4:6-Trichlorocatechol	0·67	0·72	Blue (8)	Buff	—	—	Green (7) Violet (9)
2:3:5-Trichloroquinol**	0·70	—	Violet (7)	Yellow	—	—	—
2:4:6-Trichlorophenol	0·92	0·74	Blue (8)	No colour	No colour	No colour	—
2:4:6-Trichlororesorcinol	0·64	0·94	Violet (8)	Green	Violet	Yellow	—
Trichlorophloroglucinol	0·10	—	Violet (8)	Brown	Orange	Orange	Violet

* Descending chromatography on Whatman no. 1 paper. In solvent A (benzene–acetic acid–water, 1:1:2, v/v), values are after 6 hr. and in solvent B (ethanol–*n*-butanol–3 N-ammonia–ammonium carbonate buffer, 11:40:19, v/v) after 16 hr.

† 2% ethanolic solution of 2:6-dichloroquinonechloroimide, followed by saturated $NaHCO_3$ for pH 8, saturated borax for pH 9, and 2N-Na_2CO_3 for pH > 10.

‡ Diazotized *p*-nitraniline, followed by 2N-Na_2CO_3.

§ Diazotized sulphanilic acid, followed by 2N-Na_2CO_3.

‖ 0·1% aqueous solution of Brentamine Fast Red B salt, followed by 2N-ammonia.

¶ 1% aqueous $FeCl_3$, followed by saturated $NaHCO_3$.

** This compound turns green, then violet, in dilute alkali.

to hydrolyse ethereal sulphates. It was then continuously extracted with ether for 10 hr. From this extract 2:3:4-trichlorophenol (50 mg.) was isolated as described above and identified as the benzoate, m.p. and mixed m.p. 142°. The residual urine was now made 3N with respect to HCl and boiled under reflux for 3 hr. to hydrolyse glucuronides. The phenols were extracted and distilled as before and 750 mg. of 2:3:4-trichlorophenol, m.p. 81°, were isolated and characterized as the benzoate, m.p. and mixed m.p. 142°. The total trichlorophenol isolated accounted for 17% of the dose.

The steam distillates, after the separation of 2:3:4-trichlorophenol from them, were extracted with ether to remove residual phenols. The extract was subjected to paper chromatography, the solvent system B being used. On spraying the paper with diazotized *p*-nitraniline or diazotized sulphanilic acid two spots were revealed near the solvent front and close together (R_F, 0·83 and 0·89). The faster-moving spot was light orange with diazotized *p*-nitraniline and orange with diazotized sulphanilic acid and corresponded to 3:4:5-trichlorophenol (R_F, 0·89, see Table 2). The slower spot was violet with diazotized *p*-nitraniline and light yellow with diazotized sulphanilic acid and corresponded to 2:3:4-trichlorophenol (R_F, 0·83). Thus it appeared that, although the 2:3:4-phenol is the major metabolite, the 3:4:5-phenol, which is readily distinguishable from the 2:3:4-isomer by colour reactions, is also formed in small amounts.

3:4:5-*Trichlorocatechol.* The residue from the steam distillates of the extracts described above contained non-volatile phenols. The residues from several experiments were combined, and on testing for catechols with $FeCl_3$ a positive green colour was obtained. The combined residues were extracted with ether and the extract was chromatographed in two solvents. When $FeCl_3$ was used for spraying, a catechol was detected, with R_F 0·55 in solvent A and R_F 0·76 in solvent B. These R_F values corresponded to 3:4:5-trichlorocatechol (see Table 2), and from the spot dimensions the amount present was equivalent to approx. 0·5% of the dose. The catechol fraction was therefore separated from the extract as the lead salt (Porteous & Williams, 1949) and the lead salt treated with H_2S to remove lead. The filtrate from the lead sulphide was extracted with ether to remove the catechol and the whole of the extract chromatographed on a large scale on no. 1 Whatman paper with benzene–acetic acid–water (1:1:2, v/v) as solvent. The position of the catechol on the paper was located and the catechol-containing portion of the paper excised. On elution of the excised strip with ethanol and evaporation of the eluate a small amount of the crystalline catechol was obtained. After recrystallization from light petroleum b.p. 60–80°, and then water, the catechol (5 mg. from 8 g. of trichlorobenzene) had m.p. and mixed m.p. 103° with authentic 3:4:5-trichlorocatechol.

The mercapturic acid fraction. The urine of three rabbits which had collectively received 4·5 g. of 1:2:3-trichlorobenzene was collected for 3 days, and adjusted to pH 2 with 2N-H_2SO_4. It was then continuously extracted with ether for 24 hr. The carboxylic acids in the ether extract were then transferred into 2N-$NaHCO_3$, and the bicarbonate extract was twice washed with ether to remove any contaminating phenols. This extract was then made N with respect to NaOH and refluxed for 0·5 hr. to hydrolyse the mercapturic acids to the corresponding thiophenols. The hydrolysed solution was acidified with 2N-H_2SO_4 and steam-distilled to yield trichlorothiophenol as a colourless oil

(60 mg.) which crystallized with difficulty. On oxidation of the thiophenol with ethanolic iodine solution, 3:4:5:3′:4′:5′-hexachlorodiphenyl disulphide was obtained as a colourless solid, m.p. and mixed m.p. 152° after repeated recrystallization from aqueous ethanol. The m.p. of 2:3:4:2′:3′:4′-hexachlorodiphenyl disulphide (185°) was depressed by the material from the urine. This suggests that the acid formed from 1:2:3-trichlorobenzene is 3:4:5-trichlorophenylmercapturic acid.

From 1:2:4-*trichlorobenzene*

Monophenols. Steam distillation of the ether extract of the acid (3N)-hydrolysed urine collected for 4 days from four rabbits which had collectively received 6 g. of 1:2:4-trichlorobenzene gave a liquid mixture of phenols (0·9 g.), which on benzoylation yielded 2:4:5-trichlorophenyl benzoate, m.p. and mixed m.p. 92° after repeated recrystallization from aqueous ethanol. Paper chromatography of the ether extract in solvent B and spraying with diazotized *p*-nitraniline revealed a violet spot, R_F 0·83, and an orange spot, R_F 0·84, which corresponded to synthetic 2:4:5- and 2:3:5-trichlorophenol respectively. Attempts to isolate 2:3:5-trichlorophenol in the pure state were unsuccessful, although it was present in the mixture in appreciable amounts. From a melting point–composition curve of mixtures of pure 2:4:5- and 2:3:5-trichlorophenols, the minimum m.p. (39–43°) was found at about 40% of the 2:3:5- and 60% of the 2:4:5-isomer. The solidification point of this mixture was 18°. The phenol mixture from the urine was liquid at room temperature (22°). From this evidence and from paper chromatography we conclude that the urinary phenol contained slightly more of the 2:4:5- than of the 2:3:5-isomer. The presence of 2:3:6-trichlorophenol in the extracts could not be shown.

3:4:6-*Trichlorocatechol.* The residue from the steam distillation of the extract described above, which contained the non-volatile phenols, gave a green colour with $FeCl_3$. Paper chromatography of an ether extract of this residue and spraying with $FeCl_3$ followed by 2N-$NaHCO_3$ revealed a single violet spot, R_F 0·67 in solvent A and 0·72 in B, which corresponded to 3:4:6-trichlorocatechol. From the dimensions of the spot the amount present was estimated to be about 0·2% of the dose, but the crystalline catechol was not isolated.

The mercapturic acid fraction. Steam distillation of the alkali-hydrolysed mercapturic acid fraction of the urine from three rabbits which had collectively received 4·5 g. of 1:2:4-trichlorobenzene yielded 100 mg. of trichlorothiophenol, which partly crystallized. The crystals were separated mechanically from the liquid. On oxidation with ethanolic iodine, the crystalline thiophenol yielded 2:4:5:2′:4′:5′-hexachlorodiphenyl disulphide, m.p. and mixed m.p. 136–7° after recrystallization from aqueous ethanol, and the liquid fraction gave 2:3:5:2′:3′:5′-hexachlorodiphenyl disulphide, m.p. and mixed m.p. 168° after recrystallization. The isolation of these disulphides suggests that two mercapturic acids, namely 2:4:5- and 2:3:5-trichlorophenylmercapturic acids, are metabolites of 1:2:4-trichlorobenzene.

From 1:3:5-*trichlorobenzene*

Phenols. Steam distillation of the ether extract of the acid-hydrolysed urine from a rabbit which had received 1:3:5-trichlorobenzene (1·5 g.) yielded 2:4:6-trichlorophenol (50 mg.), m.p. 67° after recrystallization from light petroleum b.p. 60–80°; the benzoate had m.p. and mixed m.p. 74°. Chromatography of the ether extract in solvent B and spraying with Gibb's reagent revealed a blue spot, R_F 0·74, corresponding to synthetic 2:4:6-trichlorophenol, which appeared to be the only metabolite of 1:3:5-trichlorobenzene. 2:4:6-Trichlororesorcinol and trichlorophloroglucinol were not detected.

The mercapturic acid fraction. Alkaline hydrolysis of the mercapturic acid fraction of the urine from three rabbits which had collectively received 4·5 g. of 1:3:5-trichlorobenzene yielded what appeared to be a trace of a thiophenol (detected by odour) on steam distillation. Oxidation with ethanolic iodine, however, gave no crystalline disulphide.

Unchanged trichlorobenzene. Six rabbits were each fed with 1·5 g. of 1:3:5-trichlorobenzene. The faeces were collected daily, homogenized with an equal volume of water, and steam-distilled. Unchanged 1:3:5-trichlorobenzene (a total of 800 mg. or 9% of the dose), m.p. and mixed m.p. 61°, was recovered from the distillate on the first 2 days after dosage. No further unchanged material was obtained on subsequent days. The urines were collected over 5 days, adjusted to pH 3 with HCl, and continuously extracted with ether for 8 hr. The ethereal extract was evaporated to dryness, suspended in N-NaOH and steam-distilled. The steam distillate contained no unchanged trichlorobenzene. Traces, however, were found in the steam distillate from acid-hydrolysed urine. Similar experiments were carried out on the faeces of rabbits receiving 1:2:3- and 1:2:4-trichlorobenzene, but in both cases none of the unchanged material was found in the faeces.

RESULTS

Table 3 shows that the major metabolites of the trichlorobenzenes are oxygen conjugates containing glucuronic acid and sulphuric acid. In the case of the 1:2:3-isomer, which appears to be the most rapidly metabolized of the three isomers, these amount to about 62% of the dose in 5 days, and after this time metabolites are not detectable in the urine. The glucuronide and ethereal sulphate excretions were found to rise to a maximum usually on the first day after dosing. None of this isomer was found unchanged in the faeces. With the 1:2:4-isomer, the oxygen conjugates amount in 5 days to about 38% of the dose, which is less than with the 1:2:3-isomer. Furthermore, the maximum excretion of conjugates was found to be reached usually on the second day after dosing, and the excretion of metabolites was still detectable after 5 days. None of the unchanged 1:2:4-isomer was found in the faeces. With the 1:3:5-isomer, the total conjugation in 5 days was much less than with the other isomers and amounted to only about 23% of the dose. The glucuronide and ethereal sulphate excretion was found to rise after the first day and remain at about the same level for the first 3 days after dosing and then progressively diminish. However, metabolites were still being excreted after 5 days and unchanged 1:3:5-

Table 3. *Quantitative excretion of metabolites of the isomeric trichlorobenzenes*

Dose fed, 0·5 g./kg. wt. of rabbit. Figures refer to percentage of dose excreted during 5 days after dosing. Figures in parentheses are mean values. Superior figures indicate the number of experiments.

	Percentage of dose excreted in the urine as					
					Trichlorophenols	
Trichlorobenzene fed	Glucuronide	Ethereal sulphate	Mercapturic acid	Total conjugation	Free	Total
1:2:3-	46–55 (50)[3]	9–13 (12)[3]	0·2–0·5 (0·3)[3]	55–69 (62)[3]	3–5 (4)[3]	64–89 (78)[4]
1:2:4-	18–33 (27)[3]	10–12 (11)[3]	0·2–0·5 (0·3)[3]	30–45 (38)[3]	1–2 (1·5)[3]	33–51 (42)[3]
1:3:5-	16–23 (20)[5]	1–5 (3)[5]	0 (0)[2]	17–28 (23)[5]	0·4–0·5 (0·5)[3]	7–13 (9)[5]

trichlorobenzene was found in the faeces. The ethereal sulphate excretion after this isomer was small and probably not significant. This is perhaps to be expected, since the only phenolic metabolite detected was 2:4:6-trichlorophenol, and this phenol is known not to form an ethereal sulphate in rabbits (Dodgson, Smith & Williams, 1950).

In all cases small amounts of free phenols were excreted, and there is an approximate agreement between the total *O*-conjugates and the conjugated phenols excreted in the case of the 1:2:3- and 1:2:4-isomers. The higher values of the total phenol as compared with the total conjugates are in part due to the fact that a mixture was estimated and the results were calculated on the basis of the extinction value of the major component phenol. With the phenols from 1:2:3-trichlorobenzene, for example, the major component, 2:3:4-trichlorophenol, has a smaller ϵ value at 246 mμ. (7100 in 0·1 N-NaOH) than the minor component, 3:4:5-trichlorophenol (ϵ, 10700 in 0·1 N-NaOH). With the 1:3:5-isomer, the total conjugates amounted to 17–28 %, but the amount of conjugated 2:4:6-trichlorophenol excreted was 7–13 %. At first it was thought that this discrepancy might have been due to the excretion of the other possible phenolic metabolites of 1:3:5-trichlorobenzene, namely 2:4:6-trichlororesorcinol or trichlorophloroglucinol. However, neither of these compounds could be detected in the urine, and the reason for the discrepancy has to be left undecided.

DISCUSSION

The trichlorobenzenes are metabolized slowly in rabbits by oxidation to monophenols, which are mainly excreted in combination with glucuronic acid and to a lesser extent with sulphuric acid. The rate at which these compounds are metabolized appears to be in the descending order 1:2:3-, 1:2:4-, 1:3:5-. In contrast with monochlorobenzene and the *o*- and *m*-dichlorobenzenes, the amounts of mercapturic acid and catechols formed are very small. With monochlorobenzene, the mercapturic acid and catechols formed account for about 20 and 27 % of the dose respectively; with the *o*- and *m*-dichlorobenzenes, the values are 5–11 and 3–4 % respectively; but with the 1:2:3- and 1:2:4-trichlorobenzenes the values are less than 0·5 % of the dose in each case. As pointed out by Azouz, Parke & Williams (1953) catechol and mercapturic acid formation appear to be related in some way. In the case of 1:3:5-trichlorobenzene the formation of a catechol derivative is not possible; mercapturic acid formation was doubtful and the amount was certainly less than 0·1 % of the dose. The orientations of the metabolites formed are summarized in Fig. 4.

Phenols

The major metabolites of the trichlorobenzenes are trichlorophenols. With the 1:2:3-isomer, the major phenol is 2:3:4-trichlorophenol (I). The other possible isomer, 3:4:5-trichlorophenol (III), is also formed, but only in small amounts. The only possible catechol, 3:4:5-trichlorocatechol, is also formed in very small quantities. The orientations of these phenols could be explained (cf. Parke & Williams, 1955) by postulating the formation of a 3:4:5-trichloro-1:2-dihydro-1:2-dihydroxybenzene (II), which could be dehydrated to form the monophenols found and dehydrogenated to form the catechol (Fig. 5). The dihydro-diol (II) on dehydration would be expected to yield mainly (I), i.e. the hydroxyl group remaining after dehydrating the diol should be located mainly at the carbon atom of 1:2:3-trichlorobenzene that is most activated in electrophilic substitutions (Badger, 1949; cf. Robinson, Smith & Williams, 1951). The major product of nitration of 1:2:3-trichlorobenzene is 2:3:4-trichloronitrobenzene (cf. Huntress, 1948).

In the case of 1:2:4-trichlorobenzene, three trichlorophenols, namely 2:4:5- (IV), 2:3:5- (VI) and 2:3:6-, are possible. 2:3:6-Trichlorophenol,

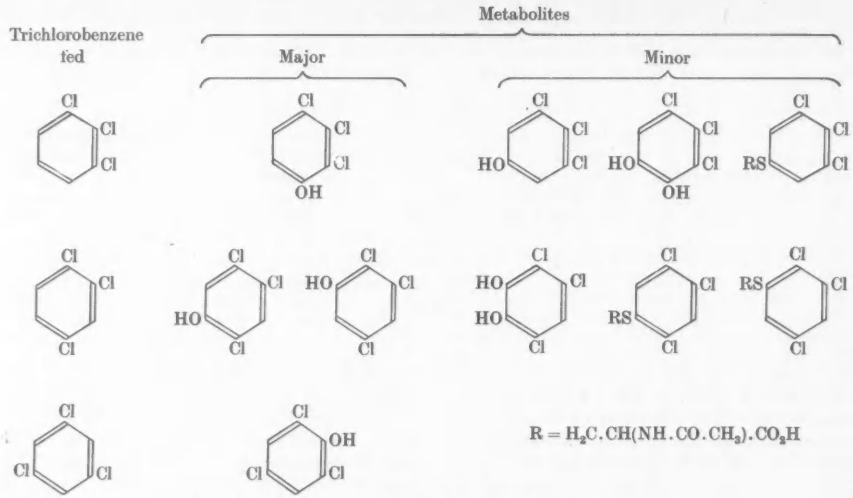

Fig. 4. Orientation of the metabolites of trichlorobenzene.

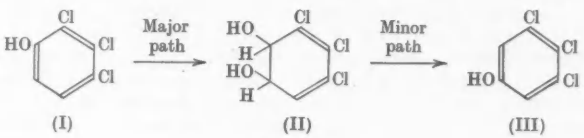

Fig. 5

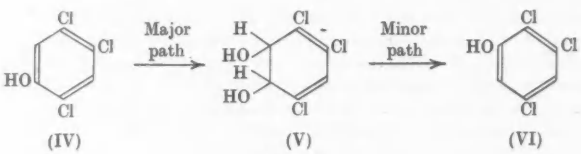

Fig. 6

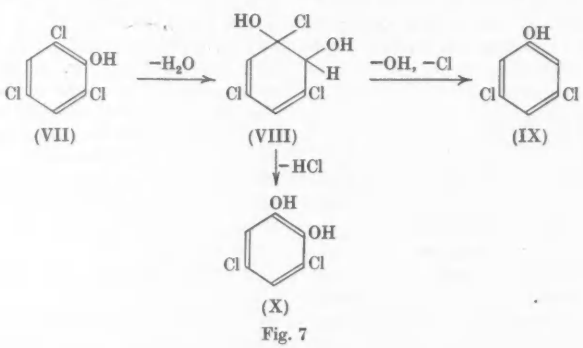

Fig. 7

however, was not found as a metabolite. The other two phenols were both found, and we believe that they were present approximately in the ratio 3:2, although only the major one (2:4:5-) was isolated in pure crystalline form. Again an intermediate 3:4:6-trichoro-1:2-dihydro-1:2-dihydroxybenzene (V) could be postulated as the precursor of these two phenols (Fig. 6). The most activated carbon atom in 1:2:4-trichlorobenzene is the one at position 5, since, on nitration with fuming nitric acid, 2:4:5-trichloronitrobenzene is formed exclusively (for references see Huntress, 1948). On these grounds one would expect (V) to yield mainly the phenol (IV), with small amounts of (VI). Thus qualitatively the orientations of the metabolic phenols and catechols of both 1:2:3- and 1:2:4-trichlorobenzenes could be explained by postulating the intermediary 1:2-dihydro-1:2-dihydroxy derivatives (II) and (V).

In the case of 1:3:5-trichlorobenzene, a 1:2-dihydrodiol at unsubstituted carbon atoms cannot be formulated. The alternative is to postulate a 1:3:5-trichloro-2-hydro-1:2-dihydroxybenzene (VIII) (Fig. 7). Dehydration of (VIII) would give (VII), the actual phenol found. Loss of HCl would give 3:5-dichlorocatechol (X), and of OH and Cl 3:5-dichlorophenol (IX), but neither of these compounds was found as metabolites of 1:3:5-trichlorobenzene. In fact, no other chlorinated phenol but (VII) was found.

1:3:5-Trichlorobenzene differs from its isomers in that the ethereal sulphate output after its administration is insignificant. In Table 4 the pK values of the monophenolic metabolites of the di- and trichlorobenzenes and the percentages of the dose of the chlorinated benzenes excreted as ethereal sulphates are given. It is to be noted that only in 2:4:6-trichlorophenol does the pK value fall below 7, and this phenol is at least ten times stronger than any other phenol given in the table. Anderton, Smith & Williams (1948) suggested that phenols with pK_a values less than 7 are not conjugated with sulphuric acid in rabbits, and Dodgson, Smith & Williams (1950) showed that 2:6-di-, 2:4:6-tri- and penta-chlorophenols (pK_a, 6·80, 6·42 and 5·26 respectively) were not excreted as ethereal sulphates, whereas chlorophenols with pK_a values higher than 7 were. On these grounds, 1:3:5-trichlorobenzene, since it is oxidized to 2:4:6-trichlorophenol, would not be expected to increase ethereal sulphate output. Since the ethereal sulphate output in this instance is practically zero, it supports the finding that 2:4:6-trichlorophenol is the only phenol formed.

Table 4. *Ethereal sulphate formation of chlorinated benzenes and the* pK_a *of the phenolic metabolites*

Chlorobenzene fed	Sulphate conjugation (% of dose)	Chlorophenol metabolites	pK_a of metabolites*
1:2-Di-	21†	3:4-Di-	8·39
		2:3-Di-	7·44
1:3-Di-	7†	2:4-Di-	7·74
		3:5-Di-	7·92
1:4-Di-	27†	2:5-Di-	7·35
1:2:3-Tri-	12	2:3:4-Tri-	7·58
		3:4:6-Tri-	7·33
1:2:4-Tri-	11	2:4:5-Tri-	7·74
		2:3:5-Tri-	7·27
1:3:5-Tri-	0	2:4:6-Tri-	6·42

* Quoted from Murray & Gordon (1935).
† From Parke & Williams (1955).

Mercapturic acids

The acetylcysteine conjugation of the trichlorobenzenes is very small, being less than 0·5% of the dose with the 1:2:3- and 1:2:4-isomers and negligible with the 1:3:5-isomer. Previous work with 1:2- and 1:3-dichlorobenzenes had shown that the orientations of the mercapturic acids from these compounds were identical with those of the major phenolic metabolites. Thus the major phenol of 1:2-dichlorobenzene was 3:4-dichlorophenol and the mercapturic acid was 3:4-dichlorophenylmercapturic acid (Azouz *et al.* 1955; Parke & Williams, 1955). This is again true of 1:2:4-trichlorobenzene, but not of the 1:2:3-isomer. In the latter case the mercapturic acid appears to be 3:4:5-trichlorophenylmercapturic acid, whose orientation is similar to that of the minor metabolic phenol (3:4:5-trichlorophenol) of 1:2:3-trichlorobenzene. The case of 1:2:4-trichlorobenzene is interesting because it appears to give rise to two mercapturic acids in roughly equal amounts, namely 2:4:5- and 2:3:5-trichlorophenylmercapturic acids (cf. Fig. 4), and these are similar in orientation to the two major phenolic metabolites. In most instances hydroxylation and mercapturic acid formation occur in the chlorinated benzenes at the most electronegative or least electropositive carbon atom, as indicated by the position of *in vitro* nitration of these compounds. The agreement, however, is not general, as is shown in Table 5. At present no *in vitro* pattern has been found which agrees entirely with the *in vivo* one.

Bromobenzene on injection into rats causes central hepatic necrosis, which can be prevented by methionine or cysteine supplements (Koch-Weser, Huerga, Yesinick & Popper, 1953). Chlorobenzene, *o*-dichlorobenzene and *m*-bromochlorobenzene, but not *p*-dibromobenzene or *p*-bromochlorobenzene, also produce liver damage in small animals. The trichlorobenzenes have only a slight effect on the liver, whilst the tetra- and hexa-derivatives are

Table 5. *Orientation of* in vivo *hydroxylation and mercapturic acid formation with chlorinated benzenes, compared with that of* in vitro *nitration*

	Position of			
	Hydroxylation *in vivo*		Mercapturic acid formation *in vivo*	Nitration *in vitro*
Compound	Mono	Di		
Chlorobenzene	4 (trace at 2)	3:4-*	4	4 (70%), 2 (30%)
1:2-Dichlorobenzene	4 (a little at 3)	3:4-†	4	4
1:3-Dichlorobenzene	4 (a little at 5)	4:5-†	4	4 (a little at 2)
1:2:3-Trichlorobenzene	4 (a little at 5)	4:5-†	5	4 (a little at 5)
1:2:4-Trichlorobenzene	5 and 6	5:6-†	5 and 6	5

* 4-Chlorocatechol is the major metabolite of chlorobenzene in rabbits.
† These catechols are minor metabolites.

without effect even in high doses (Cameron *et al.* 1937; Koch-Weser *et al.* 1953). The mono- and *o*- and *m*-di-halogenobenzenes form appreciable amounts of mercapturic acids *in vivo*, whilst the *p*-di- and the tri-halogenobenzenes form little or none. It thus appears that the toxic effects of these compounds are due to withdrawal of methionine and cysteine from the liver for mercapturic acid formation. It seems likely that since the tetra-, penta- and hexa-chlorobenzenes are relatively non-toxic they do not form mercapturic acids to any appreciable degree. This has already been found to be true for some of the tetrachlorobenzenes (unpublished data).

SUMMARY

1. A study has been made of the fate in the rabbit of oral doses (0·5 g./kg.) of the three isomeric trichlorobenzenes.

2. The 1:2:3-isomer is the most rapidly metabolized isomer. It is nevertheless slowly metabolized, 62% of the dose appearing, during 5 days after dosing, as conjugates of glucuronic (50%) and sulphuric acids (12%). The maximum excretion of these conjugates occurred on the first day after dosing. The major metabolite was 2:3:4-trichlorophenol, but small amounts of 3:4:5-trichlorophenol and traces of 3:4:5-trichlorocatechol were also formed. Small quantities (0·3%) of mercapturic acid were formed and identified as 2:3:4-trichlorophenylmercapturic acid.

3. The 1:2:4-isomer was more slowly metabolized and oxygen conjugates accounted for 38% of the dose in 5 days, the maximum occurring on the second day after dosing. Two major phenols, 2:4:5- and 2:3:5-trichlorophenol, were excreted, together with small amounts of 3:4:6-trichlorocatechol. Small amounts (0·3%) of two mercapturic acids, 2:3:5- and 2:4:5-trichlorophenylmercapturic acid, were excreted.

4. 1:3:5-Trichlorobenzene was the least rapidly metabolized of the isomers, and only about 23% of the dose was excreted as oxygen conjugates in 5 days. Practically no ethereal sulphate or mercapturic acid was formed and the only phenol detected was 2:4:6-trichlorophenol. Unchanged 1:3:5-trichlorobenzene was found in the faeces.

5. The paper chromatographic behaviour and colour reactions of 11 trichlorophenols have been recorded, together with the ultraviolet spectra of some of them.

6. Five isomeric hexachlorodiphenyl disulphides have been synthesized as reference compounds for the identification of trichlorophenylmercapturic acids.

7. The orientations of the phenolic metabolites found have been discussed in the light of current theories of metabolic hydroxylation.

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Studies on Ali-esterases and other Lipid-hydrolysing Enzymes

3. INHIBITION OF THE ESTERASES OF PANCREAS*

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Extensive studies of the substrate specificity of crude preparations of pancreatic lipase have been carried out by many investigators (cf. Ammon & Jaarma, 1950), though they usually failed to consider the fact that the pancreas contains more than one enzyme with esterase activity. The results obtained by Loevenhart & Pierce (1907) with sodium fluoride and by Platt & Dawson (1925) with albumin and edestin as inhibitors suggest that different enzymes are responsible for the hydrolysis of ethyl esters and olive oil by pancreas. Although these authors did not draw this conclusion, later experiments by Wolvekamp & Griffoen (1934) and Fodor (1946, 1950*a*, *b*) showed that the suggestion is justified.

It is generally agreed that pancreatic lipase can hydrolyse tributyrin as well as triolein (cf. Willstätter & Memmen, 1923), and recent evidence presented by Fodor (1950*a*) and Aldridge (1954) supports the assumption that the activity of pancreas preparations towards tributyrin is due largely, if not entirely, to the lipase. Fodor (1950*b*) suggested further that the hydrolysis of esters such as methyl butyrate is due largely to cholesterol esterase, an enzyme which was shown by Kelsey (1939) to be distinct from the lipase of pancreas. The methods used by Fodor (1946, 1950*a*, *b*) to distinguish these two enzymes (effect of gum acacia, bile salts, trypsin digestion and exposure to high temperatures and to alkali) did not permit of a definition of their relative substrate specificities. However, Webb (1948) has suggested that organophosphorus inhibitors would be useful tools in a study of the substrate specificity of esterases and lipases, since the lipases are relatively resistant to inhibition by these compounds. The results obtained by Aldridge (1953*a*, 1954), Mounter & Whittaker (1953) and Myers *et al.* (1955) with serum and other tissues have confirmed the value of this technique. In the present investigation we have therefore reinvestigated the specificity of the pancreas esterases, using various organo-phosphorus derivatives as esterase inhibitors.

We found that the pancreas enzymes responsible for the hydrolysis of tributyrin and methyl butyrate could indeed be readily distinguished. Further attention was paid to the hydrolysis of salicyl butyrate since Hofstee (1952*a*, *b*) reported that pancreas preparations contain two esterases capable of hydrolysing salicyl esters, neither of which is identical with lipase. Our experiments confirm the conclusions of Hofstee (1952*a*, *b*), as well as those of Fodor (1950*a*, *b*), and show that it is possible to distinguish at least three types of enzymes with esterase activity in pancreas by the use of organo-phosphorus inhibitors.

MATERIALS AND METHODS

Substrates. Many of the substrates were prepared in this laboratory by Miss J. W. Tol according to the methods described in the literature; these esters include phenyl

* Part 2: Myers, Schotte & Mendel (1955).

acetate, propionate and butyrate (Perkin, 1889), *p*-nitrophenyl acetate and butyrate (Huggins & Lapides, 1947), salicyl butyrate (Hofstee, 1952*b*), the phenyl ester of phenylacetic acid and the ethyl esters of L-tyrosine and L-leucine (Fischer, 1901). The remaining esters tested were commercial preparations from British Drug Houses Ltd. and Eastman Kodak Ltd.

Inhibitors. Diethyl *p*-nitrophenyl phosphate (E 600) and di*iso*propyl *p*-nitrophenyl phosphate (DINP) were obtained through the courtesy of Albright and Wilson, Ltd., di*iso*propyl phosphorofluoridate (DFP) was supplied by Dr B. C. Saunders, Cambridge, *NN'*-di*iso*propyl phosphorodiamidofluoridate (mipafox, Isopestox) by Dr G. S. Hartley, Pest Control Ltd., and *NN*-dimethylcarbamoyl fluoride by Dr G. Schrader, Elberfeld. Tri-*o*-cresyl phosphate (TOCP) was obtained commercially from Eastman Kodak Ltd. and methanesulphonyl fluoride was synthesized in this laboratory by Mr A. Kemp, jun., according to the method described by Davies & Dick (1932).

Manometric determination of esterase activity. Esterase activity was determined by the Warburg method at 37·5° and pH 7·4 in a medium containing 0·025 M-$NaHCO_3$ and saturated with CO_2+N_2 (5:95, v/v). Rat-pancreas suspensions were prepared with a glass Potter–Elvehjem homogenizer and diluted in the Warburg vessels so that the vessels contained 0·05–2 mg. of fresh pancreas in a fluid volume of 5 ml. for most experiments. The reaction mixture was incubated for 30 min. at 37·5°, with or without inhibitor, before adding a small volume of substrate to determine the residual esterase activity. All esters were prepared in the form of an aqueous emulsion, suspension or solution containing gum acacia such that the final concentrations of ester and gum acacia in the reaction mixture were 0·2 and 0·1% respectively. The values given for the esterase activities are corrected for the spontaneous hydrolysis of the substrate concerned.

Titrimetric determination of lipase activity. The manometric method proved unsuitable for determining the rate of hydrolysis of olive oil (triolein), since the values obtained were always very low. The titrimetric methods described for serum lipase by Cherry & Crandall (1932) and for pancreatic juice by Lips (1951) gave much more satisfactory results. The latter technique was used in most of the experiments in the present investigation. A portion (2 ml.) of a pancreas suspension containing 50–100 mg. of fresh pancreas/ml. were mixed with 2 ml. of 5% (w/v) sodium taurocholate solution and shaken with 10 ml. of olive oil to obtain an emulsion. Samples were withdrawn after incubation at 37·5° for various periods up to 4 hr., pipetted into ethanol and titrated with alkali.

The pH of the reaction mixture usually fell from 6·7 to about 5·3 within a 4 hr. period of incubation. When olive oil was replaced by tributyrin as substrate, the pH dropped much more rapidly; under these conditions, better results were obtained when the reaction mixture was buffered with phosphate. Since the rate of hydrolysis of the oil decreased appreciably with longer periods of incubation, the lipase activity was calculated on the basis of the hydrolysis occurring within the first hour. All values were corrected for a control containing pancreas suspension which had been inactivated by heating at 100°.

Martin & Peers (1953) have reported that the lipase of oat seeds exhibits maximum activity, with olive oil as substrate, in a reaction mixture which contains relatively small amounts of water. Larger amounts of oil did not improve the results obtained by the method described above with the lipase of pancreas; however, when proportionately less oil was used, a less satisfactory emulsion was obtained and the lipase activity appeared to be lower. The taurocholate was necessary to produce a finely dispersed emulsion but could be replaced by gum acacia when the method of Cherry & Crandall (1932) was applied to pancreas suspensions. Satisfactory results could also be obtained with serum as emulsifying agent when olive oil was used as substrate for the pancreas lipase; the normal sera have no appreciable activity towards olive oil under these conditions.

In several experiments on the reversibility of the inhibition by E 600, the pancreas suspension was first incubated with E 600 alone for 30 min., subsequently an equal volume of rabbit serum was added and the mixture incubated for another 30 min. before adding the taurocholate and oil. In this way any excess of E 600 in the solution was completely destroyed by the A-esterase in the rabbit serum (Aldridge, 1953*b*) before the residual lipase activity of the pancreas was determined. Control suspensions without E 600 but treated in the same way with rabbit serum exhibited a normal lipase activity towards olive oil.

Colorimetric determination of cholesterol-esterase activity

Cholesterol-esterase activity was measured colorimetrically (Sperry & Webb, 1950) with the techniques described by Yamamoto, Goldstein & Treadwell (1949). The pH of the reaction mixture, which contained 1% of sodium taurocholate and 0·4% of cholesterol ester, was maintained at 6·3–6·4. The esterase activity was usually calculated from the hydrolysis occurring within the first 3–4 hr. at 37°. Cholesteryl acetate was used as substrate in most experiments since the cholesterol esterase of pancreas acts most efficiently on the esters of the lower aliphatic acids (Swell, Cassidy & Treadwell, 1951; Swell & Treadwell, 1955). A few experiments were carried out with the same technique to determine the rate of hydrolysis of cholesteryl stearate and the rate of esterification of free cholesterol in the presence of stearic acid (Swell & Treadwell, 1950).

RESULTS

Fresh suspensions of rat pancreas exhibit a very high activity towards tributyrin and a high activity towards phenyl butyrate. The esterase activity towards phenyl butyrate, propionate and acetate is almost completely inhibited by low concentrations of all the organo-phosphorus derivatives investigated, whereas the activity of the lipase towards tributyrin is not significantly affected by low concentrations of most of these compounds (Table 1). A similar result was obtained with the methanesulphonyl and dimethylcarbamoyl fluoride derivatives (Table 1), which have been shown to act similarly to the organo-phosphorus derivatives (Myers & Kemp, 1954). The classical inhibitors for distinguishing esterase activity from lipase activity (Rona & Pavlovic, 1922) proved to be much less satisfactory; high concentrations of atoxyl do indeed inhibit the esterase activity towards phenyl

butyrate without affecting the lipase activity towards tributyrin appreciably, but quinine inhibited both enzymes equally (Table 1). As could be surmised from the results obtained by Loevenhart & Pierce (1907), the esterase activity was more sensitive than the lipase activity to inhibition by sodium fluoride (Table 1).

In confirmation of the results obtained by Aldridge (1954), E 600 exhibits a considerable inhibitory activity against the lipase of rat-pancreas suspensions (Table 1). Its *iso*propyl analogue, DINP, also exhibits some inhibitory activity. The degree of inhibition of the hydrolysis of olive oil is in all cases almost identical with that observed when tributyrin is used as substrate (see Table 2). These data confirm the conclusion of previous investigators that both substrates are hydrolysed by the same enzyme, a lipase.

As found previously (Myers, 1954), this inhibition by E 600 is progressive (Table 2) and irreversible. The data given in Tables 2 and 3 show that the inhibition of the lipase activity is not affected by a thousand-fold dilution of the incubation mixture containing E 600. Moreover, the inhibition of the pancreas lipase was found to remain unaltered for at least 4 hr. after the excess of E 600 in the reaction mixture had been removed by treatment with rabbit serum in the manner described above.

Table 1. *Sensitivity of the lipase and esterase activities of rat pancreas to various inhibitors*

Rat-pancreas suspensions were incubated for 30 min. at 37° and pH 7·4 with various concentrations of the inhibitors indicated below. Subsequently the esterase activity was measured by a manometric technique, and the negative logarithm of the molar concentration of inhibitor required to produce 50% inhibition of the activity (pI_{50}) was determined graphically. In most experiments with tributyrin, the reaction mixture contained 0·02 mg. of fresh pancreas/ml.; a concentration of 0·1 mg. of fresh pancreas/ml. was used in the experiments with phenyl butyrate as substrate.

Inhibitor	pI_{50} values: Phenyl butyrate	pI_{50} values: Tributyrin
Diethyl *p*-nitrophenyl phosphate (E 600)	7·6	5·2
Di*iso*propyl *p*-nitrophenyl phosphate (DINP)	8·4	2·6
Di*iso*propyl phosphorofluoridate (DFP)	6·7	(<3)
NN'-Di*iso*propyl phosphoro diamidofluoridate	5·5	(<3)
NN-Dimethylcarbamoyl fluoride	5·9	2·8
Methanesulphonyl fluoride	4·1	(<3)
Sodium fluoride	2·3	1·4
Quinine hydrochloride	3·2	3·2
Sodium arsanilate (atoxyl)	2·1	(<1·5)

Table 2. *Progressive inhibition of pancreas lipase on incubation with* E 600 *or DINP for various periods*

Rat-pancreas suspensions (50 mg. of fresh pancreas/ml.) were incubated at 37° with 3×10^{-4} M E 600 or 3×10^{-4} M DINP for various periods. Subsequently 2 ml. of the suspension were mixed with 2 ml. of 5% sodium taurocholate solution plus 10 ml. of olive oil and the lipase activity was determined titrimetrically as described in the text (cf. Lips, 1951). The activity of the same pancreas suspensions towards tributyrin was determined manometrically after one thousand-fold dilution in the Warburg vessel.

Inhibitor	Time of incubation at 37° (min.)	% inhibition: Olive oil	% inhibition: Tributyrin
E 600	5	36	—
	30	70	67
	60	86	84
	120	93	95
	240	94	98
DINP	30	6	12
	240	27	38

Table 3. *Inhibition of pancreas lipase by* E 600 *under various experimental conditions*

Rat-pancreas suspensions in 0·67 M phosphate buffer, pH 7·3, were incubated with various concentrations of E 600 for 30 min. before determining the lipase activity towards olive oil and tributyrin by a titrimetric or manometric method as described in the text. The results were plotted graphically in order to estimate the pI_{50} value (negative logarithm of the molar concentration of E 600 required to produce 50% inhibition).

Concn. of pancreas suspension (mg./ml.): During incubation with E 600	Concn. of pancreas suspension (mg./ml.): During measurement of activity	Technique used in measuring lipase activity	Substrate	Lipase activity (m-moles of acid/g. pancreas/hr.)	pI_{50} value with E 600
180	25·7	Titrimetric	Olive oil	21	3·5
50	7·1	Titrimetric	Olive oil	23	3·8
50	7·1	Titrimetric	Tributyrin	27	3·8
50	0·05	Manometric	Tributyrin	120	3·8
0·05	0·05	Manometric	Tributyrin	145	5·0
0·005	0·005	Manometric	Tributyrin	260	5·6

The conclusion by Aldridge (1954) that the inhibition by E 600 is reversible may be based on the fact that a given concentration of E 600 produces a much smaller degree of inhibition in a concentrated suspension than in a highly diluted suspension of the same pancreas (Table 3). This phenomenon has been observed previously with other tissues and is probably due to interaction of the organo-phosphorus derivative with the tissue constituents (Hobbiger, 1951). The same type of result is observed not only with the lipase but also with the esterase in rat pancreas which is responsible for the hydrolysis of phenyl acetate and phenyl butyrate. The amounts of E 600, DINP and DFP required to produce 50 % inhibition of this esterase are, on the average, forty times greater with a concentrated suspension (40 mg. fresh pancreas/ml.) than with a dilute suspension (0·1 mg. fresh pancreas/ml.) (see Table 1 and Fig. 1). The differences in the pI_{50} values observed with the lipase under similar conditions (Table 3) are of the same order of magnitude.

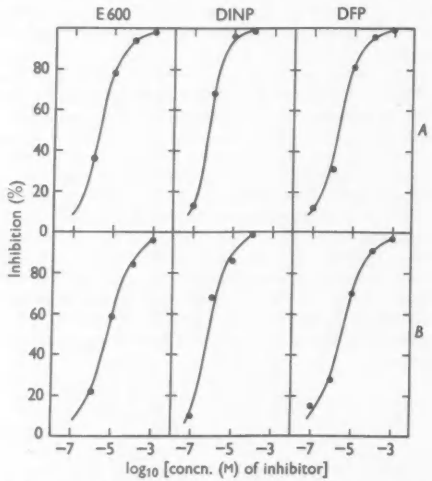

Fig. 1. Inhibition of hydrolysis of phenyl acetate (*A*) and cholesteryl acetate (*B*) by the cholesterol esterase of rat-pancreas suspensions after incubation with E 600, DINP and DFP. Pancreas suspensions (40 mg. of fresh pancreas/ml.) were incubated for 2 hr. at 37·5° with these inhibitors in various concentrations. The activity towards cholesteryl acetate was determined colorimetrically at pH 6·3 by the method of Yamamoto *et al.* (1949). The activity towards phenyl acetate was determined manometrically at pH 7·4, as described in the text, after diluting the pancreas suspension 200-fold.

The cholesterol esterase activity of pancreas suspensions is readily inhibited by the organophosphorus derivatives (Fig. 1). In this respect, therefore, the cholesterol esterase resembles the enzyme responsible for the hydrolysis of phenyl acetate, propionate and butyrate. The inhibition curves obtained with cholesteryl acetate and with phenyl acetate as substrates were similar but not identical (Fig. 1); the discrepancies observed may be due to the different techniques employed with these two substrates. No marked differences were apparent in the inhibitory effects of E 600, DINP and DFP. Further, the hydrolysis of cholesteryl acetate, the hydrolysis of cholesteryl stearate and the esterification of free cholesterol in the presence of stearic acid are all inhibited to about the same extent by DFP (Table 4); apparently these enzymic activities are all due to the same esterase (cf. Swell & Treadwell, 1955).

Table 4. *Effect of diisopropyl phosphorofluoridate on the activity of the cholesterol esterase towards various substrates*

The cholesterol esterase activity of rat-pancreas suspensions (40 mg. of fresh pancreas/ml.) was determined colorimetrically at pH 6·3, according to the technique described by Yamamoto *et al.* (1949) and Swell & Treadwell (1950).

Expt.	Substrate	Cholesterol esterase activity (μmoles/g. pancreas/hr.)	Inhibition in the presence of 10^{-4} M DFP (%)
1	Cholesteryl acetate	9·3	82
	Cholesteryl stearate	2·9	74
2	Cholesteryl acetate	10·3	79
	Cholesterol + stearic acid	1·3	80

Both the cholesterol esterase and the lipase of pancreas proved to be relatively resistant to inhibition by TOCP *in vivo*. Whereas the ali-esterases (B-type esterases) of rat serum and liver are almost completely inhibited 1 day after the administration of 50 μl. of TOCP/kg. (Mendel & Myers, 1953; Myers & Mendel, 1953), a dose of 1 ml. of TOCP/kg. intraperitoneally was required in order to produce an appreciable depression of the cholesterol-esterase activity of the pancreas (Table 5). The same dose of TOCP, which is sufficient to produce a slight diarrhoea, has less effect on the lipase activity of rat pancreas. The hydrolyses of olive oil and of tributyrin by pancreas are affected in the same way by TOCP *in vivo*, but the hydrolysis of phenyl acetate again tends to be somewhat more strongly inhibited than the hydrolysis of cholesteryl acetate (Table 5).

In these experiments (cf. Table 5), the injection of TOCP did not affect significantly the amount of free cholesterol or cholesterol ester in the serum. The total amount of cholesterol in the liver also

Table 5. *Effect of tri-o-cresyl phosphate (TOCP) injection on the lipase and cholesterol esterase of rat pancreas*

Male rats weighing approximately 250 g. were injected with 1 ml. of TOCP/kg. intraperitoneally. At various times after the injection, the rats were killed and the pancreas removed. The activity of the pancreas suspensions towards olive oil was determined titrimetrically, the activity towards cholesteryl acetate colorimetrically and the activities towards tributyrin and phenyl acetate manometrically.

Time after the injection of TOCP (days)	No. of rats	Lipase and esterase activities (m-moles acid/g. pancreas/hr.)			
		Olive oil	Tributyrin	Phenyl acetate	Cholesteryl acetate
0	15	24	290	28	0·0119
1	6	17	205	25	0·0113
3	6	20	240	10	0·0049
5	6	16	195	14	0·0090
7	6	21	255	17	0·0096
9	6	22	270	26	0·0130

Table 6. *Effect of heating on the lipase and esterase activities of rat-pancreas suspension*

A suspension containing 100 mg. of fresh pancreas/ml. was heated in a water bath at 60° for various periods. The activities towards tributyrin and phenyl butyrate were determined in the usual way with 0·01 and 0·1 mg. of pancreas/ml. respectively in the Warburg vessels. The activity towards salicyl butyrate was measured with 10 mg. of pancreas/ml. in the Warburg vessel, in the presence and in the absence of 10^{-3} M E 600; that portion of the activity which was sensitive to inhibition by E 600 is indicated under I, and the remaining portion, which was insensitive to E 600, is indicated under II.

Time of heating at 60° (min.)	Lipase and esterase activities (μl. of CO_2/mg. pancreas/hr.)			
	Tributyrin	Phenyl butyrate	Salicyl butyrate I	Salicyl butyrate II
0	7120	1380	3·8	8·0
5	4200	118	0·8	8·2
10	2980	10	0	8·8
20	1980	0	0	8·4
60	260	0	0	8·8

remained unaltered, although the relative proportion of cholesterol ester increased from 10 to 30% of the total cholesterol after injection of 1 ml. of TOCP/kg.

A third type of enzyme in rat-pancreas suspensions could be distinguished when salicyl butyrate was employed as substrate. According to Hofstee (1952*a*, *b*), the hydrolysis of salicyl esters by pancreatin preparations is due to two enzymes which he designated as esterase I and esterase II. Esterase I was readily inactivated by exposure to high temperatures, but esterase II was more resistant to heating; both enzymes could be distinguished from the lipase and from the cholinesterases. In the present investigation it was found that the activity of rat-pancreas suspensions towards salicyl butyrate was relatively low (Table 6). Approximately 30–40% of the total activity is readily inhibited by E 600, DINP or DFP in low concentrations and is quickly destroyed at 60°. This enzyme, esterase I, resembles the esterase responsible for the hydrolysis of phenyl butyrate (Table 6). The remaining portion of the activity towards salicyl butyrate was resistant to heating (Table 6) and proved to be completely insensitive to inhibition by E 600 and other organophosphorus derivatives. In both respects this second enzyme, esterase II, differs from the lipase of pancreas and resembles the A-esterase of serum.

Table 7 shows the results obtained in a preliminary investigation of the relative substrate specificities of the lipase and DINP-sensitive esterase in rat pancreas. In most cases it was not possible to demonstrate any significant activity of the esterase II towards the esters tested with the exception of salicyl butyrate. Salicyl acetate and the other acetates tested are not hydrolysed by this enzyme. A small portion of the total activity towards methyl butyrate, amounting to less than 1 μl. of CO_2/mg. pancreas/hr., was insensitive to both E 600 and DINP. However, the small portion of the total hydrolysis of phenyl butyrate which is not due to the DINP-sensitive esterase could be inhibited by E 600 in high concentrations and was therefore ascribed to the lipase.

In confirmation of the results obtained by Fodor (1950*a*, *b*), it was found that the hydrolysis of methyl butyrate and other non-glyceride esters was due mainly to the DINP-sensitive esterase of the pancreas, although the lipase does exhibit a small activity towards this ester. The lipase appears to hydrolyse triolein and tributyrin with equal facility (Table 3), although tripropionin is hydrolysed more slowly (Table 7). These results correspond with those obtained by Balls, Matlack & Tucker (1937), who found maximal activity

towards tricaproin and triheptylin. The lipase exhibits a much smaller activity towards triacetin under the experimental conditions. A large portion of the total activity towards triacetin is due to the DINP-sensitive esterase of pancreas.

Table 7. *Rate of hydrolysis of various esters by the lipase and esterase of rat pancreas*

The activities were measured manometrically at pH 7·4 and 37·5° with 0·2% (w/v) ester in the presence of 0·1% (w/v) gum acacia. The amounts of pancreas were varied between 0·01 and 10 mg. of fresh pancreas/ml., so that the rate of hydrolysis of the substrate in question was usually in the range 1–6 μl. of CO_2/min. The portion of the activity which was completely inhibited by 10^{-6}M, 10^{-5}M and 10^{-4}M DINP is given under the heading 'Esterase I'. The portion of the activity which was not inhibited by these concentrations of DINP and which was completely inhibited by 10^{-3}M E600 was ascribed to the lipase. No value is given when the activities of other enzymes towards the same substrate were so high that a reliable measurement of the activity in question was not possible.

	Enzyme activity (μl. of CO_2/mg. of pancreas/hr.)	
Substrate	Lipase	Esterase I
Tributyrin	6580	—
Tripropionin	2180	—
Triacetin	20	13
Diacetin	5	5
Monoacetin	2	3
Amyl acetate	2	16
Ethyl acetate	0·5	1·4
Methyl acetate	0·9	2
Methyl butyrate	6	50
Methyl benzoate	1	27
Benzyl acetate	105	160
Benzyl benzoate	1	70
o-Cresyl acetate	—	470
Salicyl acetate	—	1
Salicyl butyrate	—	3
Phenyl acetate	5	660
Phenyl propionate	10	1250
Phenyl butyrate	14	1220
Phenyl phenylacetate	5	90
Phenyl benzoate	1	70
p-Nitrophenyl acetate	8	1090
p-Nitrophenyl butyrate	25	1170

As could be surmised from the results obtained by Fodor (1946) and Aldridge (1954), the activity of the pancreas lipase is strongly influenced by the presence of gum acacia. This is not due simply to protection of the pancreatic lipase by gum acacia against destruction at 37·5° (cf. Child, 1945); the results were not appreciably altered when the pancreas suspension itself was incubated with gum acacia instead of adding the gum acacia to the substrate in the usual manner. The gum acacia is important for the emulsification of insoluble substrates (Aldridge, 1954), but it also influences the activity towards triacetin which is completely soluble in water at the concentration used in these experiments. The rate of hydrolysis of triacetin by the lipase in the presence of 0·2% (w/v) gum acacia was almost twice as great as in the absence of gum acacia, though the esterase activity was not appreciably altered.

DISCUSSION

The esterases in rat pancreas differ from the various types of ali-esterases found in other tissues. The ali-esterases (B-type esterases) are capable of hydrolysing both triglycerides and phenyl esters at an appreciable rate and are inhibited by low concentrations of E600, DINP and related compounds (Aldridge, 1953*a*; Mounter & Whittaker, 1953). The substrate specificity of the DINP-sensitive esterase of the pancreas (esterase I) resembles that of the A-esterase (arom-esterase) in serum and liver, i.e. it exhibits a high activity towards phenyl esters, a low activity towards ethyl acetate and triacetin and no appreciable activity towards tributyrin (Aldridge, 1953*a*, 1954; Mounter & Whittaker, 1953). However, the pancreas esterase is very sensitive to inhibition by the organophosphorus derivatives, whereas the A-esterase is not irreversibly inhibited by these compounds, and indeed hydrolyses certain of them, e.g. E600 fairly rapidly (Aldridge, 1953*b*).

It is still uncertain whether the same esterase is responsible for the hydrolysis of phenyl and cholesteryl esters by pancreas suspensions. The fact that both activities are readily inhibited by the organo-phosphorus derivatives suggests that this may be the case. Another type of cholesterol esterase may be responsible for the esterification of free cholesterol that occurs when serum or plasma is allowed to stand (Sperry, 1935). We have found that this esterification can be prevented by the presence of DFP, E600 or DINP in concentrations of 10^{-5}M and 10^{-4}M with rat serum and human serum respectively. However, we have not been able to identify this enzyme by the methods used in the present investigation; possibly the common esterase activity of this enzyme is very small in comparison with that of the other esterases present in serum. In this respect it might be noted that Byron, Wood & Treadwell (1953) have shown differences in the characteristics of the cholesterol esterases present in liver and in pancreas. Although the acetylesterase of wheat seeds has not been tested for cholesterol-esterase activity, this enzyme does not hydrolyse tributyrin rapidly and in other respects appears to resemble the DINP-sensitive esterase of pancreas (cf. Jansen, Nutting & Balls, 1948; Singer & Hofstee, 1948; Mendel, Myers, Uyldert, Ruys & de Bruyn, 1953).

The differentiation of the esterase and lipase of pancreas makes possible a better interpretation of the results of previous investigators. For example, the data on the relative rate of hydrolysis of *p*-nitrophenyl esters by pancreas preparations (Huggins & Moulton, 1948) are concerned with the DINP-sensitive esterase and not with the lipase. It is to be expected that the hydrolysis of naphthyl esters is also due to the same esterase, in which case the results of Seligmann, Nachlas & Mollomo (1949) would indicate that the injection of mecholyl and eserine liberates the DINP-sensitive esterase as well as the lipase and other enzymes from the pancreas into the blood stream. Similarly, it is of interest that a large portion of the total activity towards triacetin was due to the pancreatic esterase in these experiments; this substrate appears therefore to be unsuitable for assay of lipase activity.

The data given in Table 7 for the substrate specificity of pancreatic lipase must be regarded with some caution. In the first place, a single concentration of ester was used with all the substrates. Secondly, the activity towards tributyrin is not always directly proportional to enzyme concentration (Table 3). However, we were not able to find evidence for the presence of a lipase inhibitor in the pancreas suspensions to explain this result. A suspension heated at 70° to inactivate the lipase had no significant effect on the hydrolysis of tributyrin by fresh pancreas suspensions; moreover, the hydrolysis of tripropionin may still be directly proportional to the amount of lipase at a concentration of pancreas where this proportionality no longer exists when tributyrin is used as substrate. This problem appears therefore to be due to the experimental techniques. Despite these complications, we have been able to confirm the previous observations that pancreatic lipase acts rapidly not only on triglycerides but also on benzyl esters, and that it does not hydrolyse benzoates rapidly (Table 7), observations which have been employed as the basis for a theory concerning the mechanism of hydrolysis by pancreatic lipase (Scott, 1953).

It might be noted that the lipase of pancreas resembles that of oat seeds in many respects, i.e. both enzymes hydrolyse olive oil and tributyrin equally well (cf. Martin & Peers, 1953), are moderately sensitive to inhibition by E 600 and relatively resistant to inhibition by DFP (cf. Mendel *et al.* 1953). Since the inhibition by E 600 is progressive and irreversible, the designation of the lipase as an A-type esterase seems inadvisable, the more so in view of the fact that the substrate specificity of the lipase is completely different from that of the A-esterase of serum.

The third enzyme described, the E 600-resistant esterase in the pancreas (esterase II), also appears to differ from the A-type esterases (arom-esterases) and the B-type esterases (ali-esterases) of other tissues. It does not hydrolyse either tributyrin or phenyl butyrate rapidly and, unlike the A-esterase of serum, does not appear to hydrolyse E 600 (cf. Aldridge, 1954). The significance of this enzyme is not known. However, preliminary studies have shown that part of the hydrolysis of certain amino-acid esters, e.g. ethyltyrosine and ethyl-leucine, by rat-pancreas suspensions is also resistant to inhibition by E 600. It is possible therefore that this esterase activity may be due to one of the proteolytic enzymes found in pancreas. Although ethyltyrosine is hydrolysed by chymotrypsin under the experimental conditions (Parks & Plaut, 1953), the enzyme in question could not be either chymotrypsin or trypsin, since both of these proteolytic enzymes are inhibited by E 600 (Hartley & Kilby, 1952; Kilby & Youatt, 1954) and other organophosphorus derivatives.

SUMMARY

1. Fresh rat-pancreas suspensions contain at least three enzymes with esterase activity which can readily be distinguished by their sensitivity to organo-phosphorus inhibitors. All three enzymes differ markedly from the A-esterases and ali-esterases of serum, liver and other tissues.

2. One of these enzymes, the lipase, hydrolyses olive oil and tributyrin rapidly, has very little activity towards triacetin and phenyl esters and is relatively insensitive to inhibition by most of the organo-phosphorus derivatives. It is, however, progressively and irreversibly inhibited by diethyl *p*-nitrophenyl phosphate (cf. Aldridge, 1954).

3. One of the esterases in pancreas hydrolyses phenyl acetate, phenyl propionate and phenyl butyrate, but exhibits little or no activity towards the triglycerides. This enzyme is readily inhibited by the organo-phosphorus derivatives and other esterase inhibitors. Since the cholesterol esterase of pancreas is also inhibited by the organo-phosphorus derivatives in low concentrations, it is possible that the esterase in question may be a cholesterol esterase (cf. Fodor, 1950*b*).

4. Unlike the ali-esterases of serum and liver, neither of these enzymes can be inhibited *in vivo* by subtoxic doses of tri-*o*-cresyl phosphate.

5. A second esterase in pancreas hydrolyses salicyl esters (cf. Hofstee, 1952*a*, *b*), exhibits little or no activity towards the triglycerides and phenyl esters and is insensitive to all of the organo-phosphorus inhibitors tested. This esterase may be a proteolytic enzyme.

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